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Monoclonal Antibodies to the Extracellular Domain of
Prostate-Specific Membrane Antigen

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13. ABSTRACT (Maximum 200 Words) The purpose of this research proposal was to evaluate the potential diagnostic and therapeutic value of radiolabeled monoclonal antibodies (J591, J533, J415) specific to the extracellular domain of PSMA (PSMA _{ext}). We previously reported all the in vitro studies and some preliminary biodistribution and radioimmunotherapy studies in nude mice. This final report describes all the in vivo studies including biodistribution and radioimmunotherapy evaluations. The pharmacokinetics, biodistribution, and tumor uptake of ¹³¹ I and ¹¹¹ In-labeled MABs were performed in nude mice bearing LNCaP tumors. There were significant differences in the absolute tumor uptake (%ID/g) among the ¹³¹ I MABs. By contrast, the tumor uptake of ¹¹¹ In and ¹⁷⁷ Lu labeled MABs was similar. However, the T/B and tumor/muscle (T/M) ratios were significantly higher with ¹¹¹ In and ¹⁷⁷ Lu compared to ¹³¹ I-MABs. In addition, the ratios were significantly higher with J591 and J415 compared to that of 7E11 (antibody specific to intracellular domain of PSMA). Radioimmunotherapy studies with ¹³¹ I-J591, ⁹⁰ Y-DOTA-J591, and ¹⁷⁷ Lu-DOTA-J591 produced an unambiguous dose-response resulting in tumor shrinkage or slowing the growth over a period of 4-6 weeks. The most important finding is that this therapeutic response was specific since ⁹⁰ Y-non-specific MAB had no response. These results clearly demonstrate that radiolabeled MABs specific PSMA _{ext} are useful for RID and RIT of prostate cancer.				
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INTRODUCTION

The prostate specific membrane antigen (**PSMA**), is a well characterized integral membrane glycoprotein, expressed in a highly restricted manner by prostate epithelial cells. PSMA is expressed by a very high proportion of PC and this expression is augmented in higher grade cancers, in metastatic disease, and in hormone-refractory PC. Therefore, we hypothesized that a radiolabeled MAb specific to the extracellular domain of PSMA (PSMA_{ext}) on viable PC cells, is ideal for in vivo prostate specific targeting strategies and would provide benefits, including improved localization and greater potential for radioimmunodiagnosis (RID) and radioimmunotherapy (RIT). In 1997, Bander and his colleagues in our institution reported the development of four IgG MAbs (J591, J533, J415, E99) to the PSMA_{ext}. Immunohistochemical studies showed excellent reactivity with PC cells. The pilot studies with ¹³¹I- and ¹¹¹In-labeled MAbs and competitive binding assays (CBA) with LNCaP cells demonstrated that J591 has higher reactivity to PSMA than CYT-356 (ProstaScint, imaging agent), which binds to cytoplasmic domain of PSMA.

The purpose of Phase I research proposal was to evaluate the potential diagnostic and therapeutic value of PSMA-specific MAbs and their fragments radiolabeled with γ - (In-111), β^- (I-131, Y-90) and α emitting radionuclides (Bi-212 or Bi-213). The specific objectives were:

- To optimize radioiodination techniques of MAb and fragments with I-131 and to optimize radiolabeling of MAb and their fragments with In-111, Y-90, Bi-212 using bifunctional chelating agents such as DOTA.
- To evaluate the relationship between specific activity and immunoreactivity of radiolabeled preparations using competitive binding assays and autoradiography.
- To study the biodistribution and tumor uptake of several radiolabeled MAb preparations and to identify the most suitable preparation(s) for diagnostic and therapeutic studies.
- To evaluate and compare the therapeutic response of several radiolabeled MAb preparations in nude mice bearing PSMA positive LNCaP tumors.
- To identify the most appropriate MAb radiolabeled with a β^- emitting radionuclide for subsequent clinical trials in patients with prostate cancer (phase 2 research proposal).

The Statement of Work (SOW) in the original research proposal consisted of six different phases. We previously reported (**The First Annual Report and 18-Month Competitive Progress Report**) all the in vitro studies and some preliminary biodistribution and radioimmunotherapy studies in nude mice. This final report describes a summary of all the research effort for the entire funding period of the Phase I research proposal.

BODY

The Statement of Work (SOW) in the original research proposal consisted of six different phases. Based on experimental results, relatively minor changes were made in various tasks as originally planned, but the experimental design as originally planned was implemented. We previously reported (The First Annual Report and 18-Month Competitive Progress Report) all the in vitro studies and some preliminary biodistribution and radioimmunotherapy studies in nude mice. This final report describes a summary of all the research effort for the entire funding period of the Phase I research proposal.

Phase 1: Radiolabeling MABs and in vitro studies:

The techniques of radiolabeling MABs (J591, J415, J533) and fragments with different radionuclides (^{131}I , ^{111}In , and ^{90}Y) was optimized to achieve a specific activity of 5-10 mCi/mg and yet preserve >70% immunoreactivity. Radiolabeling MAB E99 was not successful and further work with this antibody was discontinued. Compared to DTPA method, DOTA conjugation of MABs resulted in higher stability of ^{111}In and ^{90}Y labeled complexes. An average of 5 DOTA molecules could be randomly conjugated to J591 and J415 antibodies with little apparent loss of immunoreactivity. Both these antibodies could be efficiently labeled with ^{111}In or ^{90}Y (5-10 mCi/mg). Immunoreactivity of radiolabeled antibody preparations (determined using Lindmo method) was >75%. A direct comparison of the chelate stability of ^{111}In -DTPA-7E-11 and ^{111}In -DOTA-J591 showed that ^{111}In was lost from DTPA-7E-11 with an apparent half-life of 11 hours, whereas the DOTA chelate had an apparent half-life exceeding 1000 hours (**Appendix-A; Figure-3**).

In vitro characterization of radiolabeled J591, J415 and J533 MABs:

Competitive binding studies with PSMA positive and PSMA negative cell lines (both viable and permeabilized) demonstrated very interesting and **unique** PSMA binding characteristics of radiolabeled MABs. We have demonstrated clearly that there is no cross-competition between J591, J415, and J533 MABs specific to PSMA_{ext} and 7E11 MAB that is specific to the internal domain of PSMA. We also demonstrated that J591 and J415 bind to the same epitope of PSMA while the binding site for J333 appears to be a different region (epitope) of PSMA. (**Appendix-A; Figure-4A-C**). ^{131}I -J415 could be displaced from binding to LNCaP cell membranes by both J415, J591 and not J533 (Figure 4A). ^{131}I -J591 binding could be displaced by J591 and J533, but not J415 (**Figure-4B**). ^{131}I -J591 binding could be displaced by J591, J415 and J533 (**Figure-4C**). Among these three antibodies, The IC₅₀s of J591 (3.1±1.5 nM) and J415 (1.3±0.9 nM) were lower compared to that of J533 (7.7±5.5) suggesting that the affinity of J533 to PSMA is less than that of either J591 or J415.

Saturation binding studies of ^{131}I -J591 or ^{131}I -J415 to LNCaP viable intact cells (**Appendix-A; Figure -5**) showed a characteristic high affinity ($K_d = 1.8 \pm \text{nM}$) binding of an antibody to a single class of antigen (600,000 – 800,00 sites/cell). In contrast, J533 had lower affinity ($K_d = 18 \pm \text{nM}$). In parallel studies, these three antibodies bound to a similar number of PSMA sites expressed by permeabilized (ruptured) cells. In contrast, ^{131}I -7E-11 specifically bound to only 10-15% of the PSMA sites expressed by intact cells. With ruptured cells, 7E-11 binding, however, was similar to that of J591 (**Appendix-A; Figure-6**). Similarly, autoradiographic

studies performed on LNCaP tumor sections also showed that binding of J591 and J415 is very specific to the external domain of PSMA and there was no cross competition with 7E11 (data reported in the First Annual Report).

Phase II and III: Pharmacokinetics, Biodistribution and Tumor Uptake Studies:

The pharmacokinetics, biodistribution, and tumor uptake of ^{131}I and ^{111}In - labeled MABs were performed in nude mice bearing LNCaP tumors. As a control, similar studies were performed with a radiolabeled non-specific antibody.

Tumor Model

Prostate carcinoma cell lines LNCaP, DU145 and PC3 (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640, supplemented with 10% fetal calf serum, at a temperature of 37°C in an environment containing 5% CO_2 . Prior to use, the cells were trypsinized, counted and suspended in Matrigel (Collaborative Biomedical Products, Bedford, MA). Nu/Nu BalbC mice 8-10 weeks of age were inoculated, in the right and left flanks, with a suspension of 5×10^6 LNCaP cells in Matrigel. After a period of 14-18 days, tumors (100-200 mg) had developed. In eight animals, DU145 and PC3 cells were implanted in an identical way.

Biodistribution Studies:

Tumor bearing mice were injected, via the tail vein, with 80KBq of the iodinated MAB (400 MBq/mg) in 200 μL of PBS (pH 7.4, 0.2% BSA). Groups of animals were sacrificed after 2, 4 or 6 days and the major organs and tumors recovered. These samples were weighed and counted, with appropriate standards in an automatic NaI(Tl) counter. These measured relative activity data (cpm) are background corrected and expressed as a percentage of the injected dose per gram (%ID/g). These data were also fitted with a least squares regression analysis (Microcal Origin, Northampton, MA) to determine the biological clearance of the various agents. Based on initial evaluation of organ uptakes, J415, J591 and 7E11 were labeled with ^{111}In (100MBq/mg). 80 KBq of the In-111 version of these MABs was injected in groups of animals. Subsequent handling was similar for the I-131 MAB injected animals. The derived data was used to determine the residence times for the ^{131}I and ^{111}In labeled antibodies. Using the biodistribution data of ^{111}In as a surrogate for ^{90}Y and ^{177}Lu labeled antibodies the residence times for these radiolabeled MABs were also determined.

The biodistribution of ^{131}I -J591 and ^{131}I -J415 was compared to that of a non-specific antibody (anti B1 antibody (**Table-1**). At one day post injection, tumor uptake of radiolabeled J591 and J415 is 2-3 times that of non-specific antibody while the blood activity for all the antibody preparations was similar suggesting that tumor uptake of J591 and J415 is specific. By two days post injection (**Table-2**), all the iodinated PSMA-specific MABs had similar tumor accumulations except for J533. The tumor to blood ratios at day two showed that J533 was significantly lower than J415, J591 or 7E11. Similar results were seen at day 4 (**Table-3**). Compared to radioiodinated antibodies, ^{111}In labeled antibodies show an elevated uptake in liver and spleen. At six days post injection (**Table-4**), the tumor values were similar for ^{111}In labeled J591, J415 and 7E11, but the activities in the other organs, especially the blood, varied considerably. For both ^{111}In and ^{131}I forms of J415 and J591, the blood clearance was significantly faster than 7E11, with the day six values being about 50 % those of 7E11. The

tumor uptakes of the ^{111}In labeled MABs was remarkably similar at six days post injection. The ^{131}I MAB tumor uptake was systematically lower than the corresponding ^{111}In labeled MABs and probably reflects the internalization of the MABs and their cellular metabolism. Consequently, the highest tumor/blood ratios were obtained with the ^{111}In labeled J415 and J591 at six days post injection.

There were significant differences in the absolute tumor uptake (%ID/g) among the ^{131}I MABs. On day 6, J591 ($9.6 \pm 2.2\%$) was significantly less than that of J415 (15.4 ± 2.4) and 7E11 (14.5 ± 4.8). By contrast, the tumor uptake of ^{111}In labeled MABs was similar and was approximately 17% of ID/g. However, the T/B and tumor/muscle (T/M) (**Appendix-B; Figure-1**) ratios were significantly higher with ^{111}In compared to radioiodinated antibodies. In addition, the ratios were significantly higher with J591 and J415 compared to that of 7E-11. For example, on day 6, the T/B with ^{111}In -DOTA-J591 (6.1 ± 1.8) was 2-3 times compared to that of ^{131}I -J591 (2.3 ± 0.8). In comparison, the T/B and T/B ratios with J533 (data not shown) were much lower. Significant differences in the rate of radioactivity clearance from the blood and other non-target organs was seen with $\text{J415} > \text{J591} > \text{7E11} = \text{J533}$. The tumor uptake of ^{131}I -J591 in mice bearing PSMA-negative PC3 or DU145 tumors was significantly lower ($p < 0.01$) than in mice bearing LNCaP tumors. At 4 days post injection, the PC3 tumors ($n=10$) had an uptake of $0.66 \pm 0.07\%$ ID/g and the DU145 tumors ($n=6$) $0.55 \pm 0.03\%$ ID/g compared to $11.4 \pm 1.49\%$ ID/g for LNCaP.

Preliminary studies with ^{131}I labeled J591 fragments indicated that the tumor uptake (at 48 hrs) of the labeled fragments was significantly less than that of intact antibody; 4.93 ± 1.25 with F(ab')_2 and 1.71 ± 0.53 with Fab' compared to 9.58 ± 2.16 with intact J591. While the T/B and T/M ratios were slightly higher initially compared to intact antibody, the kinetics of washout were not favorable.

Imaging studies:

Animals were injected with 2 MBq ^{111}In -DOTA-J591. At 1, 2, 3, 4 and 6 days post injection, they were sedated with ketamine/xylazine 100 mg/kg/10 mg/kg IP and imaged with a ADAC Transcam gamma camera (ADAC Laboratories, Milpitas, CA) equipped with a pinhole collimator. Data were collected for a gamma energy of 245 keV with a 20 % window for 1000 seconds using a 256 x 256 matrix. Gamma camera images of the same animal, obtained up to 6 days post injection are shown in **Appendix-B; Figure-2**. After one day, the single tumor (250 mg) is easily visualized on the right hind quarter and the blood pool and liver are observed. In the later images, the activity is seen to clear from the blood and the tumor accumulation become more intense. The biodistribution studies based on counting tissue samples at various times post injection provided more quantitative data for comparison of antibodies compared to imaging studies of mice at several times post injection. Therefore, imaging studies were not performed for all the radiolabeled antibody preparations.

^{177}Lu -DOTA-J591:

In this research project, we originally did not plan to evaluate the potential therapeutic value of ^{177}Lu antibodies. However, we wanted to study the relative advantages of this ^{177}Lu labeled

antibodies compared to that of ^{90}Y labeled antibodies. **These studies are absolutely new** and were not included in the original SOW.

For targeted radioimmunotherapy of prostate cancer, huJ591 monoclonal antibody could be labeled efficiently with several radionuclides (^{131}I , ^{90}Y , ^{177}Lu) emitting beta particles. Among the 3 potentially useful radionuclides, I-131 labeled antibody is relatively unstable in vivo due to dehalogenation while Y-90 and Lu-177 labeled antibodies are quite stable in vivo. Y-90 radionuclide has very high energy beta particles with several mm range in tissue and has been investigated in many clinical trials in the last 10 years. While Y-90 nuclide can deliver higher radiation dose to tumor, the main toxicity may be due to bone marrow. As a result, administration of optimal doses of Y-90-HuJ591 may not be possible. The higher beta energy particles may be good for bulky tumors, but it may not be necessary for small tumors. In contrast, Lu-177 has low energy beta particle with only 0.2-0.3 mm range and delivers significantly less radiation dose to marrow compared to Y-90. In addition, due to longer physical half-life, the tumor residence times are higher. As a result, higher activities (more mCi amounts) of ^{177}Lu -J591 can be administered with comparatively less radiation dose to marrow. Lu-177 has been evaluated in several clinical and pre clinical trials as a potential therapeutic agent for radioimmunotherapy of ovarian and bone cancer.

The kinetics of biodistribution and tumor uptake of ^{177}Lu -DOTA-J591 was compared to that of ^{111}In -DOTA-J591 in nude mice bearing LNCaP tumors (**TABLE-5**). At 4 days post injection, the tumor uptake of ^{177}Lu was greater than that with ^{111}In (19.3 ± 4.3 vs. 15.7 ± 1.3). The tumor/blood ratios were slightly better with ^{177}Lu . The kinetics of blood clearance and tumor uptake of ^{177}Lu -DOTA-J591 was compared to both ^{111}In -DOTA-J591 and ^{131}I -J591 (**Appendix-B; Figure-3A and 3B**). While the blood activity of these three tracers was similar, the tumor uptake of ^{177}Lu -DOTA-J591 was significantly greater compared to other two tracers. These results clearly suggest the potential advantages of ^{177}Lu labeled antibodies for RIT studies.

Phase IV: RIT of Prostate Cancer: Studies in tissue culture:

Internalization and cellular processing of radiolabeled antibodies was studied in order to understand the relative advantages of ^{111}In labeled MABs over ^{131}I labeled MABs for RIT studies. LNCaP cells in a petri dish were incubated with ^{131}I and ^{111}In labeled J591 and J415 antibody preparations. The cells were incubated for 1 hr with radiolabeled J591 or J415. The unbound activity was removed and the labeled cells washed to remove any unbound radioactivity and fresh medium was added. Over the next 2 days, the % of cell bound activity was determined at several time points. The kinetics of wash out and the net cell associated activity was determined. With both antibodies, the net retention of ^{131}I activity was 30% compared to 70-90% retention with ^{111}In (**Appendix-A; Figure-7**). With J415, however, the kinetics of washout of both ^{131}I and ^{111}In activity from the cell was faster compared to radiolabeled J591. These studies suggest that the intracellular metabolism of these two MABs is different. In addition, MABs labeled with ^{90}Y would be more effective for radiotherapy, since the net retention of radioactivity is greater with ^{111}In compared to ^{131}I -MABs.

In a different study, the kinetics of uptake of ^{111}In labeled J591 and J415 was compared to that of 7E-11. LNCaP cells were incubated with ^{111}In labeled antibody preparations and % of added activity associated with cells at various times over the next 2 days was determined. With J591

and J415, the initial cellular uptake rate of ^{111}In activity was 10-20 times faster than that of 7E-11. Over the next 2 days, significantly more ^{111}In activity was associated with cells with J591 compared to J415. In contrast, the no significant uptake of ^{111}In activity was seen with 7E-11 antibody (**Appendix-A; Figure-9**).

Phase V and VI: RIT of Prostate Cancer: Studies in nude mice with LNCaP tumors

Based on in vitro and in vivo characterization of radiolabeled MAb specific to PSMAext, 4 (four) β - emitting radiolabeled antibody formulations (^{131}I -J591, ^{90}Y -DOTA-J591, ^{90}Y -DOTA-J415, ^{177}Lu -DOTA-J591) have been selected for evaluating the therapeutic efficacy of RIT in nude mice bearing LNCaP tumors.

Method:

Over a period of 18 months, 6 experiments were performed evaluating the efficacy of RIT with Radiolabeled MAb (**Table-6**). Nude mice bearing LNCaP tumors (50-100 mg or 400-800 mg) were divided into several groups (6-8 mice group). In each experiment, the test groups of mice received different doses of radiolabeled MAb by the tail vein. Some groups of mice received repeat injections of the dose 2-3 times over a period of 2 months. Animals were periodically weighed and tumor dimensions determined over a period of 2-4 months. The response to the treatment was compared to a control group of mice that received no treatment. Mice were sacrificed if the tumor burden exceeded 10% of body mass or if the body mass decreased by 20% compared to baseline value. Typically, the control group of mice experienced uncontrolled tumor growth and a steady decline in body mass. These animals had a median survival time of 6 weeks. The mice that received radiolabeled MAb preparation, showed a delay in tumor growth or a reduction in tumor size.

RIT experiment 1: 4 groups of mice (5 mice/group) with 250-400 mg of tumors were included in this study. (Table-6). The control group received no treatment. The three test groups (2-4) received ^{131}I -J591; 100 μCi by the tail vein, 100 μCi intraperitoneally and 300 μCi by the tail vein. Compared to untreated controls, anti-tumor effect of ^{131}I -J591 was dependent on the dose. At 100 μCi dose, there was some delay and reduction in tumor size. But at 300 μCi dose, all the mice had a significant reduction (>30%) in tumor size. 2/5 mice in the control group died in 4 weeks while the remaining survived more than 6 weeks. With I-131 there was no improvement in % survival rate. However, mice that received 100 μCi dose intraperitoneally survived more than 6 weeks (**Appendix-B; Figure-4**).

RIT experiment 2: 4 groups of mice (5 mice/group) with 250-400 mg of tumors were included in this study. (Table-6). The control group received no treatment. Group-2 received ^{131}I -J591 (300 μCi); groups-3 received a single 30 μCi dose of ^{90}Y -DOTA-J591 while group-4 received 2 doses (day 0 and day 21). Compared to the control group both ^{131}I -J591 and ^{90}Y -DOTA-J591 showed a 20-30% reduction in tumor size (**Appendix-B; Figure-5A**). In the control group, there was a gradual reduction (20% within 6-8 weeks) in the total body mass associated with the uncontrolled tumor growth. Similar reduction in body mass was seen with a single 30 μCi dose of ^{90}Y -DOTA-J591. However, mice that received a second injection of the treatment dose showed a gradual increase in body mass. With ^{131}I -J591, there was some recovery of body mass, but the mice died within 8-10 weeks (**Figure-5B**).

RIT experiment 3: 5 groups of mice (5-7 mice/group) with 250-400 mg of tumors were included in this study. (**Table-6**). The control group received no treatment. A second control group received a 30 μ Ci dose of ^{90}Y -DOTA-F23 (a non-specific antibody). Three other groups of mice received 3 different doses of ^{90}Y -DOTA-J591 (30, 60, and 90 μ Ci). The mice in these three groups received repeat injections at the same dose level 3 times (day 0, 28, and 56). The mice in the second control group that received a non-specific antibody also had an uncontrolled tumor growth similar to the mice that received no treatment. The anti-tumor effect (decrease in tumor size) of ^{90}Y -DOTA-J591 was dose dependent; 30% at 30 μ Ci, 50-70% at 60 μ Ci, and >70% reduction at 90 μ Ci dose level (**Appendix-B; Figures-6A and 6B**). Mice that received 30 and 60 μ Ci doses showed a significant prolongation of survival compared to control groups. 90 μ Ci dose has a shorter survival and appears to be more toxic than 30-60 μ Ci dose levels. In the control group, there was a gradual reduction (20% within 6-8 weeks) in the total body mass associated with the uncontrolled tumor growth. Mice that received repeated treatment doses of 30-60 μ Ci of ^{90}Y -DOTA-J591 showed a gradual increase in total body mass (**Figure-6C**).

RIT experiment 4: 3 groups of mice with LNCaP tumors (400-800 mg) were injected with 3 different doses (150, 200, 300 μ Ci) of ^{90}Y -DOTA-J591. All the mice received only a single treatment dose. There was a significant reduction in tumor size at all 3 dose levels. However, at 150 μ Ci dose level, tumors started growing again after 5 weeks. In contrast, most of the mice at 200-300 μ Ci dose level died within 3-4 weeks following treatment suggesting that these dose levels are associated with increased toxicity (**Appendix-B; Figure-7**).

RIT experiment 5: In this study, the anti-tumor effect of ^{90}Y -DOTA-J591 was compared to that of ^{90}Y -DOTA-J415. Mice with LNCaP tumors (150-400mg) were divided into 3 groups (n=7/group). The control group received no treatment. Group-2 received a total of 3 doses (60 μ Ci/dose) of ^{90}Y -DOTA-J591 over a period of 2 months (day 0, 35, 63). Group-3 received a total of 3 doses (60 μ Ci/dose) of ^{90}Y -DOTA-J415 over a period of 2 months (day 0, 35, 63). Compared to controls, both J591 and J415 radiolabeled MAbs preparations had a significant anti-tumor response. The % survival with both antibodies was significantly greater compared to controls. However, there is not much difference between these two antibodies (**Appendix-B; Figure-8**).

RIT experiment 6: In this study, the anti-tumor effect of ^{177}Lu -DOTA-J591 was studied. 3 groups of mice (n = 6-7/group) with LNCaP tumors (100-200 mg) were injected with a single dose of 100, 200, and 400 μ Ci of ^{177}Lu -DOTA-J591. A control group received no treatment. With 100 μ Ci, there was a delay in tumor growth. But at 200-400 μ Ci dose levels, the anti-tumor effect was significant compared to controls. 6-8 weeks following treatment, tumors started growing slowly. Compared to control mice, there was a greater survival rate at 100-200 μ Ci dose levels, but at 400 μ Ci dose appears to be toxic (**Appendix-B; Figure-9A**). There was a gradual reduction (20% within 6-8 weeks) in the total body mass of mice in the control group. In contrast, mice treated with 200 μ Ci of ^{177}Lu , showed no reduction in the body mass. But at 400 μ Ci dose level, 50% of the mice had a gradual decline in the body mass and died within 3-6 weeks (**Figure-9B**).

Table 1. Uptake of Radiolabeled mAbs (%ID/g) and Tumor/non/Tumor ratios in Nude Mice Bearing LNCaP Tumors at 1 day post injection

Organ	¹³¹ I-B1 (n=4)		¹³¹ I-J415 (n=3)		¹³¹ I-J591 (n=8)	
	mean	±stdev	mean	±stdev	mean	±stdev
Blood	10.10	± 1.30	11.10	± 1.9	12.90	± 2.6
Heart	2.86	± 0.51	3.39	± 0.98	4.06	± 1.07
Lung	4.30	± 0.20	5.37	± 1.32	5.25	± 1.20
Liver	2.17	± 0.28 ^{ab}	3.31	± 0.55 ^a	4.11	± 0.92 ^b
Kidney	2.29	± 0.28	2.83	± 0.50	2.77	± 0.71
Stomach	1.54	± 0.31 ^b	2.04	± 0.40	2.82	± 0.85 ^b
Sm. Intestine	1.15	± 0.05	1.01	± 0.22	1.14	± 0.25
Lg. Intestine	0.38	± 0.07 ^b	0.57	± 0.03	0.93	± 0.38 ^b
Muscle	0.86	± 0.11	0.74	± 0.30	0.60	± 0.52
Thyroid	16.70	± 12.0	7.73	± 5.31 ^c	15.20	± 1.6 ^c
Spleen	-----		3.36	± 0.46	3.15	± 0.62
Tumor	4.41	± 0.80 ^a	12.2	± 3.24 ^a	8.55	± 3.66
T/Blood	0.45	± 0.14 ^a	1.11	± 0.13 ^{ac}	0.70	± 0.28 ^c
T/Muscle	5.15	± 0.70 ^{ab}	18.8	± 8.2 ^a	10.60	± 6.0 ^b
T/Liver	2.08	± 0.54 _a	3.73	± 0.58 ^a	2.27	± 1.23
T/Spleen	-----		3.67	± 0.61 ^c	2.45	± 0.40 ^c

Notes:

a: p<0.05 for ¹³¹I-B1 vs. ¹³¹I-J415

b: p<0.05 for ¹³¹I-B1 vs. ¹³¹I-J591

c: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J591.

Table 2. Biodistribution of Radiolabeled MABs (%ID/g) and Tumor to Non/Tumor Ratios in Nude Mice Bearing LNCaP Tumors at 2 days post injection.

Organ	¹³¹ I-J415 (n=8)	¹³¹ I-J533 (n=4)	¹³¹ I-J591 (n=8)	¹³¹ I-7E11 (n=7)	¹¹¹ In-J415 (n=4)	¹¹¹ In-J591 (n=4)	¹¹¹ In-7E11 (n=4)
Blood	8.44 ± 2.16 ^a	12.80 ± 1.1 ^{ad}	8.57 ± 2.04 ^d	10.80 ± 3.5	6.12 ± 0.62 ^{gh}	8.98 ± 2.10 ^g	7.22 ± 0.46 ^h
Heart	2.71 ± 0.66 ^{ac}	4.82 ± 1.47 ^{ad}	2.78 ± 0.62 ^{df}	3.92 ± 0.99 ^{cf}	2.87 ± 0.44	3.10 ± 0.36	2.72 ± 0.81
Lung	4.38 ± 0.92	5.08 ± 0.45	4.65 ± 1.77	4.68 ± 0.54	4.15 ± 0.99 ^{gh}	5.89 ± 0.30 ^{gi}	4.64 ± 0.27 ^{hi}
Liver	2.56 ± 0.63 ^a	3.78 ± 0.50 ^{ade}	2.71 ± 0.50 ^d	2.96 ± 0.52 ^e	5.18 ± 1.07 ^{gh}	7.68 ± 0.50 ^{gi}	4.49 ± 0.51 ^{hi}
Kidney	2.05 ± 0.41 ^b	3.37 ± 0.39 ^{de}	2.11 ± 0.57 ^{bd}	2.16 ± 0.88 ^e	4.21 ± 0.07 ^{gh}	5.25 ± 0.63 ^{gi}	2.55 ± 0.27 ^{hi}
Stomach	1.37 ± 0.37	1.11 ± 0.34	1.46 ± 0.40	1.17 ± 0.26	1.16 ± 0.29	0.73 ± 0.18	0.92 ± 0.37
Sm Intestine	1.01 ± 0.37	0.84 ± 0.07	0.86 ± 0.18	0.94 ± 0.09	1.39 ± 0.06	1.32 ± 0.26 ⁱ	1.06 ± 0.19 ⁱ
Lg Intestine	0.50 ± 0.21	0.43 ± 0.13	0.54 ± 0.13 ^f	0.39 ± 0.06 ^f	0.76 ± 0.13 ^g	0.98 ± 0.06 ^g	0.70 ± 0.13
Muscle	0.70 ± 0.19 ^a	1.13 ± 0.26 ^{ad}	0.62 ± 0.19 ^{df}	0.95 ± 0.24 ^f	0.83 ± 0.09	0.67 ± 0.10	0.83 ± 0.37
Thyroid	18.90 ± 5.9 ^a	30.20 ± 8.3 ^{ade}	15.70 ± 10.57 ^{df}	19.4 ± 4.1 ^{ef}	2.19 ± 0.72	2.47 ± 0.37 ⁱ	1.67 ± 0.19 ⁱ
Spleen	2.47 ± 0.62	2.60 ± 0.29	2.88 ± 0.89	2.40 ± 0.45	4.48 ± 1.11	5.36 ± 1.25	3.94 ± 1.13
Tumor	13.00 ± 5.1	7.38 ± 0.97 ^d	11.20 ± 2.9 ^d	11.70 ± 4.3	11.3 ± 1.0 ^h	13.6 ± 2.8 ⁱ	9.3 ± 1.52 ^{hi}
T/Blood	1.57 ± 0.42 ^{ac}	0.58 ± 0.04 ^{ade}	1.43 ± 0.57 ^d	1.08 ± 0.18 ^{ee}	1.83 ± 0.33	1.62 ± 0.72	1.34 ± 0.24
T/Liver	5.19 ± 1.35 ^a	1.96 ± 0.18 ^{ade}	4.37 ± 1.15 ^d	3.89 ± 0.92 ^e	2.20 ± 0.52	1.76 ± 0.22	2.16 ± 0.44
T/Spleen	5.45 ± 1.69 ^a	2.84 ± 0.32 ^a	3.85 ± 1.55	3.73 ± 1.55	2.59 ± 0.76	2.39 ± 0.86	2.59 ± 0.92
T/Muscle	19.20 ± 6.0 ^{ac}	6.80 ± 1.56 ^{ade}	20.0 ± 6.9 ^{df}	12.3 ± 2.2 ^{cef}	13.4 ± 1.4	21.10 ± 7.4	13.80 ± 7.8

Notes:

- a: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J533 b: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J591 c: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-7E11
d: p<0.05 for ¹³¹I-J533 vs. ¹³¹I-J519 e: p<0.05 for ¹³¹I-J533 vs. ¹³¹I-7E11 f: p<0.05 for ¹³¹I-J591 vs. ¹³¹I-7E11
g: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-J519 h: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-7E11 i: p<0.05 for ¹¹¹In-J591 vs. ¹¹¹In-7E11.

Table 3. Biodistribution of Radiolabeled MAbs (%ID/g) and Tumor to Non/Tumor Ratios in Nude Mice Bearing LNCaP Tumors at 4 days post injection.

Organ	¹³¹ I-J415 (n=9)	¹³¹ I-J533 (n=4)	¹³¹ I-J591 (n=7)	¹³¹ I-7E11 (n=8)	¹¹¹ In-J415 (n=4)	¹¹¹ In-J591 (n=7)	¹¹¹ In-7E11 (n=4)
	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev
Blood	6.11 \pm 0.97 ^{ac}	10.3 \pm 0.48 ^{ade}	5.96 \pm 1.61 ^d	7.72 \pm 1.79 ^{ce}	4.42 \pm 0.78	4.78 \pm 0.85	5.69 \pm 1.00
Heart	2.01 \pm 0.54 ^a	2.87 \pm 0.71 ^{ad}	1.70 \pm 0.16 ^{fd}	2.59 \pm 1.03	1.84 \pm 0.45	1.82 \pm 0.37	1.58 \pm 0.49
Lung	3.51 \pm 0.85	4.49 \pm 1.05	3.35 \pm 0.97	3.80 \pm 0.76	3.72 \pm 0.50	3.40 \pm 0.32	4.03 \pm 1.03
Liver	1.97 \pm 0.40 ^a	2.56 \pm 0.18 ^{ae}	2.06 \pm 0.46	1.93 \pm 0.33 ^e	5.47 \pm 0.50h	7.66 \pm 2.44 ⁱ	4.39 \pm 0.45 ^{hi}
Kidney	1.94 \pm 0.60 ^b	2.27 \pm 0.55 ^{de}	1.37 \pm 0.24 ^{bd}	1.66 \pm 0.33 ^e	5.22 \pm 0.57h	5.39 \pm 1.27	3.81 \pm 2.08
Stomach	0.96 \pm 0.55	0.98 \pm 0.21	1.06 \pm 0.36	0.96 \pm 0.23	1.35 \pm 0.37 ^g	0.80 \pm 0.15 ^g	1.02 \pm 0.35
Sm Intestine	0.62 \pm 0.29	0.76 \pm 0.13 ^d	0.53 \pm 0.13 ^{fd}	0.77 \pm 0.08	1.69 \pm 0.19 ^{gh}	1.31 \pm 0.19 ^{gi}	0.94 \pm 0.06 ^{hi}
Lg Intestine	0.32 \pm 0.16 ^c	0.31 \pm 0.04 ^e	0.34 \pm 0.10 ^f	0.48 \pm 0.10 ^{ce}	0.88 \pm 0.06h	0.81 \pm 0.20 ⁱ	0.50 \pm 0.07 ^{hi}
Muscle	0.62 \pm 0.37	0.93 \pm 0.28 ^d	0.48 \pm 0.24 ^d	0.72 \pm 0.27	0.60 \pm 0.03	0.55 \pm 0.34	0.51 \pm 0.13
Thyroid	26.6 \pm 17.9	33.7 \pm 6.9 ^e	26.5 \pm 26.2	21.3 \pm 7.9 ^e	1.82 \pm 0.70	1.90 \pm 0.16 ⁱ	1.29 \pm 0.69 ⁱ
Spleen	2.43 \pm 1.04 ^a	2.33 \pm 0.38 ^a	2.33 \pm 0.72	1.99 \pm 0.69	4.63 \pm 1.38	4.43 \pm 0.89	3.88 \pm 1.51
Tumor	17.0 \pm 6.6 ^a	7.29 \pm 2.5	11.4 \pm 4.21	12.1 \pm 5.3	16.7 \pm 2.6	15.7 \pm 3.5	16.2 \pm 4.29
T/Blood	2.80 \pm 0.70 ^{ac}	0.72 \pm 0.29 ^{ad}	2.14 \pm 0.53 ^d	1.58 \pm 0.79 ^c	3.17 \pm 0.37	3.35 \pm 0.39	2.83 \pm 0.51
T/Liver	9.07 \pm 3.19 ^a	2.81 \pm 0.85 ^{ad}	6.41 \pm 2.89 ^d	6.32 \pm 3.41	2.32 \pm 0.16	2.19 \pm 0.47 ⁱ	3.71 \pm 1.11 ⁱ
T/Spleen	7.70 \pm 3.81 ^a	3.06 \pm 0.65 ^{ade}	5.71 \pm 2.21 ^d	5.91 \pm 1.67 ^e	2.50 \pm 0.11 ^{gh}	3.67 \pm 0.71 ^g	4.32 \pm 0.85 ^h
T/Muscle	30.7 \pm 11.3 ^{ac}	7.85 \pm 1.25 ^{ade}	29.9 \pm 10.3 ^{df}	18.1 \pm 8.0 ^{ce}	21.8 \pm 2.4 ^h	40.1 \pm 22.6	32.1 \pm 4.9 ^h

Notes:

- a: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J533
 b: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J591
 c: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-7E11
 d: p<0.05 for ¹³¹I-J533 vs. ¹³¹I-J519
 e: p<0.05 for ¹³¹I-J533 vs. ¹³¹I-7E11
 f: p<0.05 for ¹³¹I-J591 vs. ¹³¹I-7E11
 g: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-J519
 h: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-7E11
 i: p<0.05 for ¹¹¹In-J591 vs. ¹¹¹In-7E11.

Table 4. Biodistribution of Radiolabeled MABs (%ID/g) and Tumor to Non/Tumor Ratios in Nude Mice Bearing LNCaP Tumors at 6 days post injection.

Organ	¹³¹ I-J415 (n=5)	¹³¹ I-J591 (n=8)	¹³¹ I-7E11 (n=8)	¹¹¹ In-J415 (n=4)	¹¹¹ In-J591 (n=8)	¹¹¹ In-7E11 (n=4)
	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev	mean \pm stdev
Blood	3.95 \pm 0.99 ^b	4.42 \pm 1.74 ^c	7.13 \pm 1.90 ^{bc}	2.63 \pm 0.47 ^e	2.52 \pm 0.56 ^f	4.16 \pm 0.41 ^{ef}
Heart	1.57 \pm 0.47 ^b	1.42 \pm 0.60 ^c	2.46 \pm 0.46 ^{bc}	1.30 \pm 0.10	1.28 \pm 0.24	2.42 \pm 1.49
Lung	2.61 \pm 1.00	2.29 \pm 0.91 ^c	3.52 \pm 0.59 ^c	3.07 \pm 0.67	2.47 \pm 0.65	3.20 \pm 0.16
Liver	1.74 \pm 0.91	1.31 \pm 0.34 ^c	2.01 \pm 0.49 ^c	4.69 \pm 0.61 ^d	6.08 \pm 0.83 ^{df}	4.37 \pm 0.46 ^f
Kidney	1.36 \pm 0.62	1.20 \pm 0.51	1.65 \pm 0.39	4.67 \pm 0.45 ^e	4.53 \pm 0.87 ^f	2.85 \pm 0.26 ^e
Stomach	0.54 \pm 0.19	0.47 \pm 0.15	0.47 \pm 0.23	0.44 \pm 0.20	0.46 \pm 0.17	0.31 \pm 0.07
Sm Intestine	0.47 \pm 0.15	0.35 \pm 0.09 ^c	0.52 \pm 0.14 ^c	0.93 \pm 0.04 ^e	0.84 \pm 0.13 ^f	0.64 \pm 0.08 ^{ef}
Lg Intestine	0.30 \pm 0.16	0.24 \pm 0.07 ^c	0.38 \pm 0.12 ^c	0.71 \pm 0.13	0.57 \pm 0.10	0.64 \pm 0.06
Muscle	0.30 \pm 0.06 ^b	0.33 \pm 0.18 ^c	0.74 \pm 0.31 ^{bc}	0.35 \pm 0.11	0.30 \pm 0.10	0.36 \pm 0.08
Thyroid	28.5 \pm 13.1	35.1 \pm 18.4	29.3 \pm 10.6	1.66 \pm 0.22 ^{de}	0.85 \pm 0.36 ^{df}	1.38 \pm 0.25 ^{ef}
Spleen	1.37 \pm 0.25	1.74 \pm 0.72	1.74 \pm 0.38	4.32 \pm 1.32	3.36 \pm 0.61	3.14 \pm 0.60
Tumor	15.4 \pm 2.4 ^a	9.58 \pm 3.2 ^{ac}	14.5 \pm 4.8 ^c	17.7 \pm 3.1	17.4 \pm 3.5	18.7 \pm 2.2
T/Blood	4.49 \pm 1.81 ^{ab}	2.28 \pm 0.77 ^a	2.21 \pm 1.33 ^b	6.78 \pm 0.78 ^e	6.07 \pm 1.79	4.57 \pm 0.31 ^e
T/Liver	11.7 \pm 5.5	7.21 \pm 1.80	7.38 \pm 2.40	3.79 \pm 0.47 ^e	3.22 \pm 0.52 ^f	4.33 \pm 0.85 ^{ef}
T/Spleen	9.69 \pm 3.73	6.90 \pm 2.20	8.89 \pm 3.84	4.57 \pm 2.33	5.14 \pm 0.95	6.52 \pm 2.32
T/Muscle	56.1 \pm 11.2 ^{ab}	34.1 \pm 15.9 ^a	21.8 \pm 12.9 ^b	52.1 \pm 11.2 ^d	64.3 \pm 5.9 ^d	55.6 \pm 12.8

a: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-J591 b: p<0.05 for ¹³¹I-J415 vs. ¹³¹I-7E11 c p<0.05 for ¹³¹I-J591 vs. ¹³¹I-7E11
d: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-J591 e: p<0.05 for ¹¹¹In-J415 vs. ¹¹¹In-7E11 f: p<0.05 for ¹¹¹In-J591 vs. ¹¹¹In-7E11.

TABLE 5. **Biodistribution and Tumor Uptake of ^{177}Lu -DOTA-J591:**
Comparison with ^{111}In -DOTA-J591.

Organ	% Injected Dose/g with					
	<u>^{177}Lu-DOTA-J591</u>			<u>^{111}In-DOTA-J591</u>		
	Day 1	Day 2	Day 4	Day1	Day 2	Day 4
Tumor (T)	11.2 \pm 0.7	15.9 \pm 1.7	19.3 \pm 4.3	10.2 \pm 0.7	13.6 \pm 1.4	15.7 \pm 1.3
Blood (B)	12.2 \pm 0.6	8.9 \pm 0.4	4.3 \pm 0.8	11.1 \pm 1.5	9.9 \pm 1.1	4.8 \pm 0.3
Liver	6.1 \pm 0.8	4.7 \pm 0.4	4.1 \pm 0.4	8.9 \pm 0.7	7.7 \pm 0.3	7.7 \pm 0.9
Kidney	5.1 \pm 0.8	4.1 \pm 0.2	3.3 \pm 0.2	7.7 \pm 0.5	5.3 \pm 0.3	5.4 \pm 0.5
Spleen	2.6 \pm 0.5	3.3 \pm 0.2	3.3 \pm 0.6	4.8 \pm 0.4	5.4 \pm 0.6	4.4 \pm 0.3
Muscle(M)	0.75 \pm 0.04	0.65 \pm 0.02	0.77 \pm 0.29	1.37 \pm 0.29	0.67 \pm 0.05	0.55 \pm 0.13
T/B	0.92	1.8	4.5	0.92	1.4	3.3
T/M	14.9	24.5	25.0	7.5	20.3	28.5

TABLE 6: RIT of Prostate Cancer: Studies in nude mice with LNCaP tumors

RIT expt.#.	Group-1	Group-2	Group-3	Group-4	Group-5
1.	Control: (untreated)	^{131}I -J591(100 μCi)	^{131}I -J591 (100 μCi , ip)	^{131}I -J591 (300 μCi)	
2.	Control: (untreated)	^{131}I -J591(300 μCi)	^{90}Y -DOTA-J591 (35 μCi)	^{90}Y -DOTA-J591 (35 μCi) (2 doses)	
3.	Control: (untreated)	^{90}Y -DOTA-F23 (30 μCi) (Control: non-specific MAb)	^{90}Y -DOTA-J591 (30 μCi) (3 doses)	^{90}Y -DOTA-J591 (60 μCi) (3 doses)	^{90}Y -DOTA-J591 (90 μCi) (3 doses)
4.	No control	^{90}Y -DOTA-J591 (150 μCi)	^{90}Y -DOTA-J591 (200 μCi)	^{90}Y -DOTA-J591 (300 μCi)	
5.	Control: (untreated)	^{90}Y -DOTA-J591 (60 μCi) (3 doses)	^{90}Y -DOTA-J415(60 μCi) (3 doses)		
6.	Control: (untreated)	^{177}Lu -DOTA-J591(100 μCi)	^{177}Lu -DOTA-J591 (200 μCi)	^{177}Lu -DOTA-J591 (400 μCi)	

For RIT studies, radiolabeled antibody dose was injected in the **tail vein**; only in one group (experiment 1 group-3, dose was given intraperitoneally)
In some studies, the mice received 2 or 3 repeated dose injections over a period of 2 months

KEY RESEARCH ACCOMPLISHMENTS:

1. We have optimized the techniques of radiolabeling MAbs with ^{131}I , ^{111}In , ^{90}Y , and ^{177}Lu in order to achieve high specific activities (5-10mCi/mg) with out significant loss of immunoreactivity.
2. We have clearly demonstrated that MAbs J591, J415 and J533 bind specifically to the extracellular domain of PSMA_{ext}. We have also identified that J591 and J415 compete for binding to PSMA, while J533 competes with J591 but not J415. In addition, we have clearly documented that 7E-11 MAb (specific to intracellular portion of PSMA) does not inhibit the binding of J591, J415 and J533 to PSMA positive LNCaP tumor cells.
3. In nude mice with LNCaP tumors, we demonstrated that the tumor uptake of radiolabeled J591 and J415 is very specific. The absolute tumor uptake and tumor/blood ratios are much greater with ^{111}In or ^{177}Lu labeled antibodies compared to ^{131}I labeled antibodies.
4. In nude mice with LNCaP tumors, we have demonstrated that the anti-tumor effect of ^{131}I -J591, ^{90}Y -DOTA-J591 and ^{177}Lu -DOTA-J591 MAbs is dependent on the dose of radionuclide administered. 60-90 μCi of ^{90}Y and 200 μCi of ^{177}Lu labeled J591 have a significant anti-tumor response with minimal toxicity. We have also demonstrated that the anti-tumor effect of radiolabeled J591 is specific since ^{90}Y -labeled non-specific antibody did not have any anti-tumor effect.
5. We have identified that both J415 and J591 are promising MAbs for the targeting of viable PSMA-expressing tumor tissue. ^{111}In -DOTA-J591 (or J415) is potentially useful for non-invasive imaging (radioimmunodiagnosis) of tumor tissue. ^{90}Y -DOTA-J591 and ^{177}Lu -DOTA-J591 MAbs may have significant potential for radioimmunotherapy of prostate cancer.
6. Based on this Phase I research data, we applied for Phase II of the Idea Development of the PCRP. In July 2000, we were awarded funding for the Phase II research proposal to perform a dose escalation trial with ^{90}Y -DOPTA-J591 in patients with prostate cancer.

REPORTABLE OUTCOMES:

1. Vallabhajosula S, Kostakoglu L, Goldsmith S.J, Bastidas D, Navarro V Gomez D, Bander NH. Monoclonal antibody J591 specific to the extracellular domain of PSMA: A new agent for radioimmunodiagnosis (RID) and radioimmunotherapy. J Nucl Med 1998;39(5 Suppl):77P-78P (Abstract).
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CONCLUSIONS:

1. The MAb J591, J415 and J533, specific to the extracellular domain of PSMA were all radiolabeled with I-131, In-111, Y-90, and Lu-177 without any significant loss of immunoreactivity. Both in vitro and in vivo studies clearly demonstrated that these MAb bind specifically to extracellular epitopes of PSMA and do not cross-react with the intracellular epitope that binds specifically 7E11 MAb (ProstaScint). J415 and J591 bind to the same epitope of the extracellular domain of PSMA while J533 binds to a different epitope.
2. The biodistribution studies in nude mice with LNCaP tumors clearly demonstrated that the specific tumor uptake of radiolabeled J591 and J415. There were significant differences in tumor uptake among the ^{131}I MAb. By contrast, the tumor uptake of ^{111}In labeled MAb was similar. The absolute tumor uptake of ^{177}Lu -DOTA-J591 was slightly higher than that of ^{111}In -DOTA-J591. The T/B and T/M are higher with ^{111}In and ^{177}Lu compared to that of ^{131}I labeled J591 MAb.
3. The kinetics of wash out and the net cell retention of ^{131}I and ^{111}In labeled MAb was significantly different. The net retention with ^{111}In (70-80%) was much greater than with ^{131}I (30%).
4. In nude mice with LNCaP tumors, ^{131}I -J591, ^{90}Y -DOTA-J591, and ^{177}Lu -DOTA-J591 produced an unambiguous dose-response resulting in tumor shrinkage or slowing the growth over a period of 4-6 weeks. The most important finding is that this therapeutic response was specific since ^{90}Y -non-specific MAb had no response. The anti-tumor effect of ^{177}Lu -DOTA-J591 (at 200 μCi dose level) appears to be similar to that with ^{90}Y -DOTA-J591 (at 60 μCi dose level). But the toxicity with ^{177}Lu appears to be less compared to that with ^{90}Y and ^{131}I .
5. These results clearly demonstrate that radiolabeled MAb specific to the external domain of PSMA are useful for RID and RIT of prostate cancer.

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APPENDIX- A

In Vitro Characterization of Radiolabeled Monoclonal Antibodies Specific for the Extracellular Domain of Prostate-specific Membrane Antigen¹

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a well-characterized cell surface antigen expressed by virtually all prostate cancers (PCas). PSMA has been successfully targeted *in vivo* with ¹¹¹In-labeled 7E11 monoclonal antibody (mAb; ProstaScint; Cytogen, Princeton, NJ), which binds to an intracellular epitope of PSMA. This work reports the *in vitro* characterization of three recently developed mAbs that bind the extracellular domain of PSMA (PSMA_{ext}). Murine mAbs J415, J533, J591, and 7E11 were radiolabeled with ¹³¹I and evaluated in competitive and saturation binding studies with substrates derived from LNCaP cells. J415 and J591 were conjugated to 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid labeled with ¹¹¹In. The uptake and cellular processing of these antibodies were evaluated in viable LNCaP cells. All four mAbs could be labeled with ¹³¹I up to a specific activity of 350 MBq/mg with no or little apparent loss of immunoreactivity. Competition assays revealed that J415 and J591 compete for binding to PSMA_{ext} antigen. J533 bound to a region close to the J591 binding epitope, but J533 did not interfere with J415 binding to PSMA. mAb 7E11 did not inhibit the binding of J415, J533, or J591 (or *vice versa*), consistent with earlier work that these latter mAbs bind PSMA_{ext} whereas 7E11 binds the intracellular domain of PSMA. Saturation binding studies demonstrated that J415 and J591 bound with a similar affinity (K_d s 1.76 and 1.83 nM), whereas J533 had a lower affinity (K_d , 18 nM). In parallel studies, all four mAbs bound to a similar number of PSMA sites expressed by permeabilized cells (1,000,000–1,300,000 sites/cell). In parallel studies performed with viable LNCaP cells, J415, J533, and J591 bound to a similar number of PSMA sites (*i.e.*, 600,000–800,000 sites/cell), whereas 7E11 bound only to a subpopulation of the available PSMA sites (95,000 sites/cell). This apparent binding of 7E11 to viable cells can be accounted for by a 5–7% subpopulation of permeabilized cells produced when the cells were trypsinized and suspended. Up to five DOTA chelates could be bound to either J415 or J591 without compromising immunoreactivity. A comparison of the cellular uptake and metabolic processing of the ¹³¹I- and ¹¹¹In-labeled antibodies showed a rapid elimination of ¹³¹I from the cell and a high retention of ¹¹¹In. All four mAbs recognized and bound to similar numbers of PSMA sites expressed by ruptured LNCaP cells (*i.e.*, the exposed intracellular and extracellular domains of PSMA). By comparison to J415 and J591, J533 had a lower binding affinity. Both J415 and J591 recognized and bound to the same high number of PSMA sites expressed by intact LNCaP. By contrast, 7E11 bound to fewer sites expressed by intact LNCaP cells (*i.e.*, the exposed extracellular domain of PSMA). Both J415 and J591 are promising mAbs for the targeting of viable PSMA-expressing tissue with diagnostic and therapeutic metallic radionuclides.

INTRODUCTION

PCa³ is the most frequently diagnosed cancer and the second most common cause of cancer mortality in United States males (1). Many groups have studied mAbs for *in vivo* diagnosis and therapy of PCa (3–8), but the only successful application to date has been the targeting of PSMA for *in vivo* imaging. PSMA is a type II membrane protein that is expressed by virtually all PCas (9, 10). Unlike other prostate-related antigens, such as prostate-specific antigen, prostatic acid phosphatase, and prostate secretory protein, PSMA is an integral membrane protein, and therefore, it is not appreciably released into the circulation. PSMA expression has been shown to be up-regulated in both poorly differentiated (11), advanced PCas (9) and after androgen-deprivation therapy (12). Interestingly, PSMA is expressed on the tumor vascular endothelium of other carcinomas and sarcomas (13, 14) but not on normal vascular endothelium, making it also potentially useful as an antibody-mediated diagnostic and therapeutic target across the full spectrum of solid tumors.

Currently, an ¹¹¹In-labeled form of the 7E11 murine mAb is approved by the Food and Drug Administration (ProstaScint) for the clinical detection of recurrent and metastatic prostate cancer in soft tissue (15). 7E11 mAb binds to the intracellular portion (NH₂ terminus) of the PSMA antigen and, as such, does not bind viable cells (13, 14). It is believed that successful imaging with ProstaScint results from mAb binding to antigen exposed in dead or dying cells within some tumor sites (16, 17). Early clinical trials using ⁹⁰Y-labeled 7E11 resulted in no objective or biochemical (prostate-specific antigen) remissions (7).

Recently, a series of mAbs to PSMA_{ext} has been characterized and reported (13, 14, 18). In this current study, we report on the *in vitro* evaluation of radiolabeled forms of these antibodies against PSMA_{ext} and the selection of interesting candidates for *in vivo* evaluation of their diagnostic and therapeutic potential.

MATERIALS AND METHODS

Murine mAbs J415, J533, and J591 were produced as described earlier (13). Purified 7E11 was generously provided by Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA). ¹³¹I and ¹¹¹In were purchased from Norton International (Kanata, Ontario, Canada). ⁹⁰Y was purchased from New England Nuclear (Boston, MA). DOTA was purchased from Macrocytics, Inc. (Richardson, TX).

LNCaP cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640, supplemented with 10% FCS, at a temperature of 37°C in an environment containing 5% CO₂. Prior to use, the cells were trypsinized, counted, and suspended in serum-free medium. LNCaP cells were permeabilized by adding methanol at –80°C to the cells. The cells were maintained at –20°C for 20 min before the methanol was removed, and the cells were rehydrated by washing four times with PBS (with 5 mM Ca²⁺ and 5 mM Mg²⁺) over 20 min.

Cell membranes were prepared by lysing the cells with a Polytron in a

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³ The abbreviations used are: PCa, prostate cancer; mAb, monoclonal antibody; PSMA, prostate-specific membrane antigen; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; BSA, bovine serum albumin; TLC, instant TLC; HPLC, high-performance liquid chromatography; DTPA, diethylenetriaminepentaacetic acid.

hypotonic buffer [1 mM Na₂CO₃ (pH 7.4) with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). Large fragments were removed by centrifuging at 2000 × g. The supernatant was centrifuged at 150,000 × g for 2 h, and the pelleted membranes were resuspended in PBS, aliquoted, and frozen at -70°C until required.

Radioiodination

The four murine mAbs were radiolabeled with ¹³¹I using the Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) method (19). Briefly, 10-ml glass tubes were coated with 50 µg of Iodogen by adding 100 µl of a 0.5 mg/ml solution of Iodogen (Pierce, Rockford, IL) in chloroform. The chloroform was removed by blowing a gentle stream of sterile nitrogen into the tube for 30 min before the tubes were sealed and stored in the dark. The iodination reaction was initiated by adding between 4 and 40 MBq of ¹³¹I (0.01 M NaOH) to 0.08 mg of mAb in 0.1 ml of ice-cold PBS. This reaction mixture was allowed to react for 5 min on ice before being loaded onto a 10-ml Biogel-P6 column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 1% BSA in PBS. Once the reaction mixture was loaded onto the column, it was washed with 2 ml of 1% BSA PBS before the main ¹³¹I-labeled mAb fraction was eluted with 2 ml of 1% BSA PBS. The amount of free iodine in the ¹³¹I-labeled mAb preparations was evaluated using instant TLC with a silica gel impregnated glass fiber support and a mobile phase of isotonic saline. Briefly, a portion of the ¹³¹I-labeled mAb was spotted on a 10-cm ITLC-SG strip (Gelman Sciences, Ann Arbor, MI) and developed in isotonic saline. Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at an R_f of 0.5. The two portions were assayed for radioactivity, and the radiochemical purity determined using the following equation: radiochemical purity = activity between R_f 0 and 0.5/total activity in strip.

Antibody Conjugation

J415 and J591 antibodies were modified with DOTA by an analogous method to that used by Lewis *et al.* (20). This method uses the direct coupling of one of the four carboxylic acid groups of DOTA to the primary amines present in the protein structure (Fig. 1). Twenty-five mg of antibody were concentrated in a M_w 30,000 Microsep centrifugal concentrator (Pall Filtron, Northborough, MA) and washed with 5 × 4 ml of 1% DTPA (pH 5.0) over a period of 24 h. The antibody buffer was then changed to 0.1 M phosphate (pH 7.0) using the same centrifugal technique. An active ester of DOTA was created by dissolving 146 mg of DOTA (0.361 mmol) and 36 mg of *N*-hydroxysuccinimide (0.313 mmol) in 2 ml of water and adjusting the pH to 7.3 with NaOH, prior to the addition of 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. This reaction mixture was cooled on ice for 1 h before being added to the J591 solution. The resultant DOTA-antibody conjugate was separated from the excess DOTA and other reactants by repeated washing with 0.3 M NH₄OAc (20 × 4 ml) and centrifugal concentration.

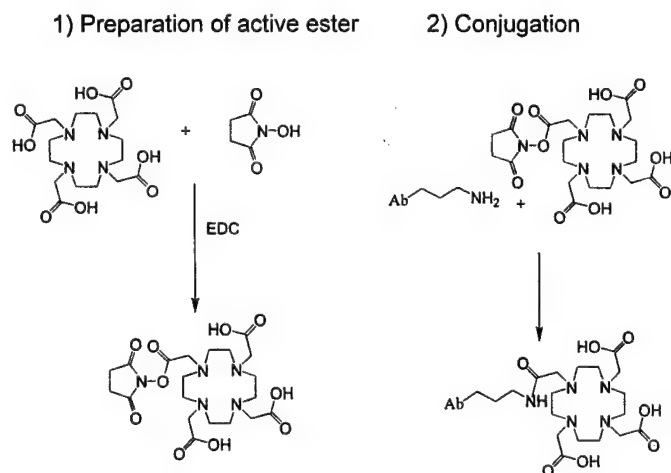


Fig. 1. Two-step conjugation of DOTA to free amines displayed by either J415 or J591. The first step used *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to create an active ester with DOTA. In the second step, the unpurified active ester is allowed to react with the monoclonal antibody.

Assay of Binding Site Number

DOTA-J591 conjugate concentration was assayed by determining the UV absorption at 280 nm. Two 50-µl aliquots of DOTA-J591 were mixed with either 20 or 40 µl of a 1.30 mM solution of InCl₃ (0.01 M HCl) spiked with a tracer amount of ¹¹¹In. The mixture was incubated at 37°C for 16 h and then analyzed by ITLC, using a silica gel-impregnated glass fiber 10-cm strip (ITLC-SG; Gelman) and an eluant of 1% DTPA (pH 6.0). Antibody-bound activity remains at the origin, and free In³⁺ moves with the solvent front as an [In-DTPA]²⁻ complex. The relative amounts of In³⁺ and In-DOTA-J591 were determined by cutting the ITLC strip at a R_f of 0.5 and counting the two halves with a Na(Tl)I detector. The number of binding sites was calculated by considering the molar reaction ratio between In and DOTA-mu-J591, and the observed ratio of ¹¹¹In and ¹¹¹In-DOTA-mu-J591 was detected.

¹¹¹In and ⁹⁰Y Labeling of DOTA Conjugate

Radiolabeling of DOTA-J591 with ¹¹¹In was achieved by adding the radionuclide (in dilute HCl) to the ammonium acetate-buffered DOTA-J591. Briefly, a mixture composed of 20 µl of ¹¹¹InCl₃ (300 MBq), 0.01 M HCl, and 400 µl of DOTA-J591 (4 mg/ml; 0.3 M NH₄OAc, pH 7) was allowed to react at 37°C for 20 min. The reaction mixture was then separated on a 20-ml Biogel-P6 column equilibrated with 4 × 10 ml of sterile 1% HSA in PBS. After the reaction mixture was loaded onto the column, it was washed with an additional 5 ml of 1% HSA PBS before the main ¹¹¹In-DOTA-J591 fraction was eluted with 3 ml of 1% HSA PBS. A similar procedure was used for radiolabeling with ⁹⁰Y, but an incubation time of 5 min was used, and the labeling mixture included 50 mM ascorbic acid.

Free ¹¹¹In in the radiolabeled DOTA-J591 preparations was determined using the ITLC method with a silica gel-impregnated glass fiber support and a mobile phase of 1% DTPA (pH 5.5). A portion of the radiolabeled DOTA-J591 was spotted on a 10-cm ITLC-SG strip and developed in 1% DTPA (pH 5.5). Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at a R_f of 0.5. The two portions were assayed for radioactivity, and the radiochemical purity was determined using the equation described earlier.

Chelate Stability Studies

¹¹¹In-labeled DOTA-J591 and DTPA-7E11 were mixed with an equal volume of 50 mM DTPA and maintained at 37°C. Periodically, samples were removed and spotted on a 10-cm ITLC-SG strip and developed in 0.9% NaCl. Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at a R_f of 0.5. The two portions were assayed for radioactivity, and the amount of intact chelate was determined using the equation described earlier.

Binding Studies

Immunoreactivity. The immunoreactivity of the ¹³¹I- and ¹¹¹In-labeled mAb preparations was assessed by the method of Lindmo *et al.* (21), which extrapolates the binding of the radiolabeled antibody at an infinite excess antigen. Briefly, six test solutions were prepared (in duplicate) and contained 20,000 cpm of the radioiodinated antibody, and increasing amounts of membranes were prepared from LNCaP cells in a total test volume of 250 µl of PBS (0.2% BSA, pH 7.4). The solutions were incubated at 37°C for 45 min prior to being filtered through a glass membrane filter and washed with ice-cold 10 mM Tris-0.9% NaCl buffer. Filters were counted in a gamma counter with standards representing the total radioactivity added. Data were then plotted as the reciprocal of the substrate concentration (X axis) against the reciprocal of the fraction bound (Y axis). The data were then fitted according to a least squares linear regression method using Origin software (Microcal Software, Inc., Northampton, MA). The Y intercept gave the reciprocal of the immunoreactive fraction. A similar method using intact or permeated LNCaP cells and centrifugal isolation of the cells gave the same results.

Competitive Binding Studies. Competitive binding studies were performed with each of the radioiodinated antibodies and the four unlabeled antibodies using either LNCaP tumor sections or membranes derived from LNCaP tumors. Acetone fixed and frozen 10-µm tumor sections were soaked in Tris buffer [170 mM (pH 7.4), with 2 mM CaCl₂ and 5 mM KCl] for 15 min and then washed with Tris buffer (170 mM, pH 7.4). The sections were then

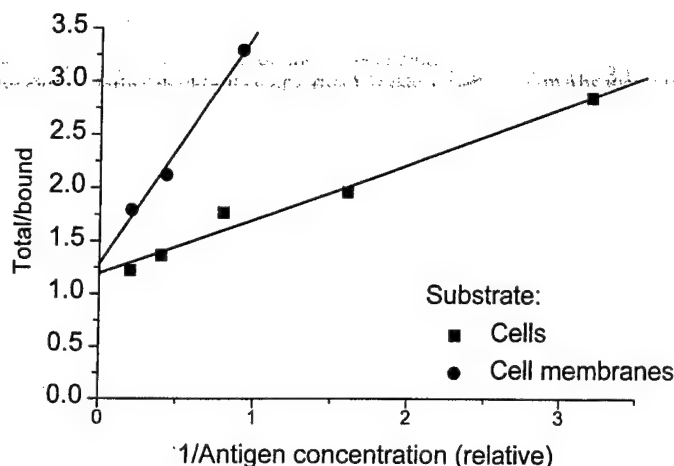


Fig. 2. Lindmo immunoreactivity testing of radiolabeled antibodies. Twenty thousand cpm of radiolabeled antibody is incubated with increasing amounts of LNCaP cell membranes at 37°C for 45 min. The membranes are then isolated by filtration through a glass fiber filter and then counted in a gamma counter. Total/bound data are then plotted as a function of the reciprocal antigen concentration. The Y intercept gives the reciprocal of the immunoreactivity. The assay, when performed with either intact LNCaP cells (■) or LNCaP cell membranes (●), gives the same immunoreactivity of ~80% for this labeled mAb.

incubated with the radioiodinated antibodies in the presence of 100 nM concentrations of each of the unmodified mAbs for 1 h at 4°C. Sections were washed three times with PBS (0.2% BSA) and once with Tris buffer (170 mM, pH 7.4) prior to being fixed with acetone and exposed with BioMax film (Kodak). The assay using the membranes typically used 50 µg of membranes, 10 fmol of iodinated antibody, and amounts of competing antibody from 0.25 fmol to 25 pmol in a 250-µl volume of PBS (0.2% BSA). Membranes were isolated as described above, and data were analyzed by a least squares regression method and Origin software (Microcal Software, Inc.) was used to determine the IC_{50} s.

Saturation Binding Studies. Saturation binding studies were performed with each of the radiolabeled antibodies using substrates of intact and permeated LNCaP cells. Briefly, 10 test solutions were prepared (in duplicate) and they contained increasing amounts of the radioiodinated antibodies, 500,000 LNCaP cells in a total volume of 250 µl of PBS (0.2% BSA, pH 7.4). The solutions were incubated at 4°C for 1 h and centrifuged and washed twice with ice-cold PBS (0.2% BSA). For each concentration of radiolabeled antibody, nonspecific binding was determined in the presence of 100 nM of the unmodified antibody. The data were analyzed with a least squares regression method (Origin; Microcal Software, Inc.) to determine the K_d and B_{max} values, and a Scatchard transformation was performed.

Internalization and Cellular Processing of J415 and J591

LNCaP cells were plated in 8-cm² Petri dishes and allowed to grow until confluent. One µCi of either the ¹³¹I- or ¹¹¹In-labeled forms of J415, J591, or 7E11 (~0.1–0.2 µg) were added to cells and allowed to incubate for 1 h. The medium was then removed, and the cells were washed once with fresh media. One ml of fresh medium was added, and the cells were incubated for up to 2 days at 37°C. Triplicate samples were periodically removed, and the medium was isolated. Surface bound activity was stripped and collected with an ice-cold acid wash (100 mM acetic acid, 100 mM glycine, pH 3.0). The cells were then treated with 1 ml of a 1% solution of Triton X-100 (containing 5 µg/ml each of antipain, pepstatin, and leupeptin as well as 1 mM phenylmethylsulfonyl fluoride) and kept at on ice for 20 min. The resultant suspension was then centrifuged, and the three samples were counted with a gamma counter. The medium and supernatants were also analyzed by ITLC and size exclusion HPLC to determine the amounts of free iodine produced or the size of the radioactive species created.

RESULTS

Radiolabeling and Quality Control. The radioiodination yield for the four mAbs was typically 70–80%, and the amounts of free iodine

in the purified mAbs was <0.3%. Specific activities of 350 MBq/mg were routinely achieved. The immunoreactivities of the ¹³¹I-labeled mAbs were determined by extrapolating the binding of a fixed amount of ¹³¹I-labeled mAb to an infinite amount of PSMA (Lindmo method; Fig. 2). This method gave immunoreactivities of >75% for all mAbs tested. When labeling conditions were increased to produce specific activities >350 MBq/mg, the immunoreactivity was compromised.

An average of five DOTA molecules could be randomly conjugated to J591 and J415, with little apparent loss of immunoreactivity. Conjugation of an average of eight DOTA molecules to J591 resulted in a 20% reduction in immunoreactivity. A 90% incorporation of ¹¹¹In could be achieved within 15 min. A 90% incorporation of ⁹⁰Y could be achieved within 5 min. Using the DOTA-J591 conjugate with an average of five DOTA molecules attached, specific activities of 280 MBq ¹¹¹In/mg DOTA-J591 and 360 MBq ⁹⁰Y/mg DOTA-J591 were achieved.

¹¹¹In-Chelate Stability Studies. A direct comparison of the chelate stability of ¹¹¹In-DTPA-7E11 and ¹¹¹In-DOTA-J591 showed that ¹¹¹In was lost from DTPA-7E11 with an apparent half-life of 11 h, whereas the DOTA chelate had an apparent half-life exceeding 1000 h (Fig. 3).

Competitive Binding Studies: Membranes. Radiolabeled J415 could be displaced from binding to LNCaP cell membranes by both J415 and J591 but not J533 (Fig. 4A). The J415 mAb had a mean IC_{50} of 1.5 nM (± 0.9 ; $n = 6$), and J591 had a mean IC_{50} of 6.6 nM (± 4.5 ; $n = 6$). Similarly, ¹³¹I-labeled J533 could be displaced by J533 and J591 but not by J415 (Fig. 4B). In these studies, J533 had a mean IC_{50} of 2.3 nM (± 1.5 ; $n = 3$), and J591 had a mean IC_{50} of 1.7 nM (± 1.3 ; $n = 3$). Finally, ¹³¹I-labeled J591 could be displaced by J415, J533, and J591 (Fig. 4C). The observed IC_{50} s were 1.3 nM (± 0.9 ; $n = 6$) for J415, 7.7 nM (± 5.5 ; $n = 6$) for J533, and 3.1 nM (± 1.5 ; $n = 6$) for J591. The 7E11 mAb did not inhibit the binding of J415, J533, or J591 (or vice versa). These data are consistent with earlier data (11) that J415, J533, or J591 bind to the extracellular domain of PSMA, whereas 7E11 binds to the intracellular domain of PSMA (13).

Saturation Binding Studies. The saturation binding curves generated were characteristic of high affinity binding of an antibody to a single class of antigen. These studies, performed with intact LNCaP cells (Fig. 5), demonstrated that J415 and J591 bound with a similar

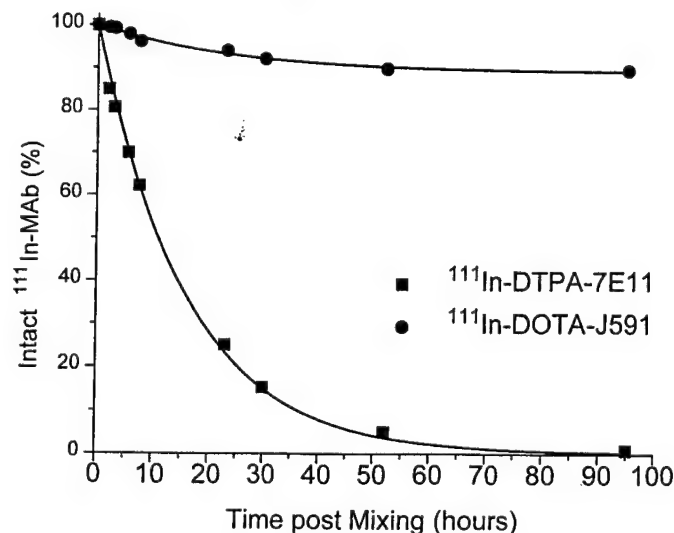


Fig. 3. Stability of ¹¹¹In-labeled mAbs. The ¹¹¹In-labeled mAbs were mixed with 50 mM DTPA, and samples were removed and analyzed by ITLC over the next 4 days. ¹¹¹In-DOTA-J591 (●) had an apparent half-life of >1000 h, and ¹¹¹In-DTPA-J591 (■) had an apparent half-life of 11 h.

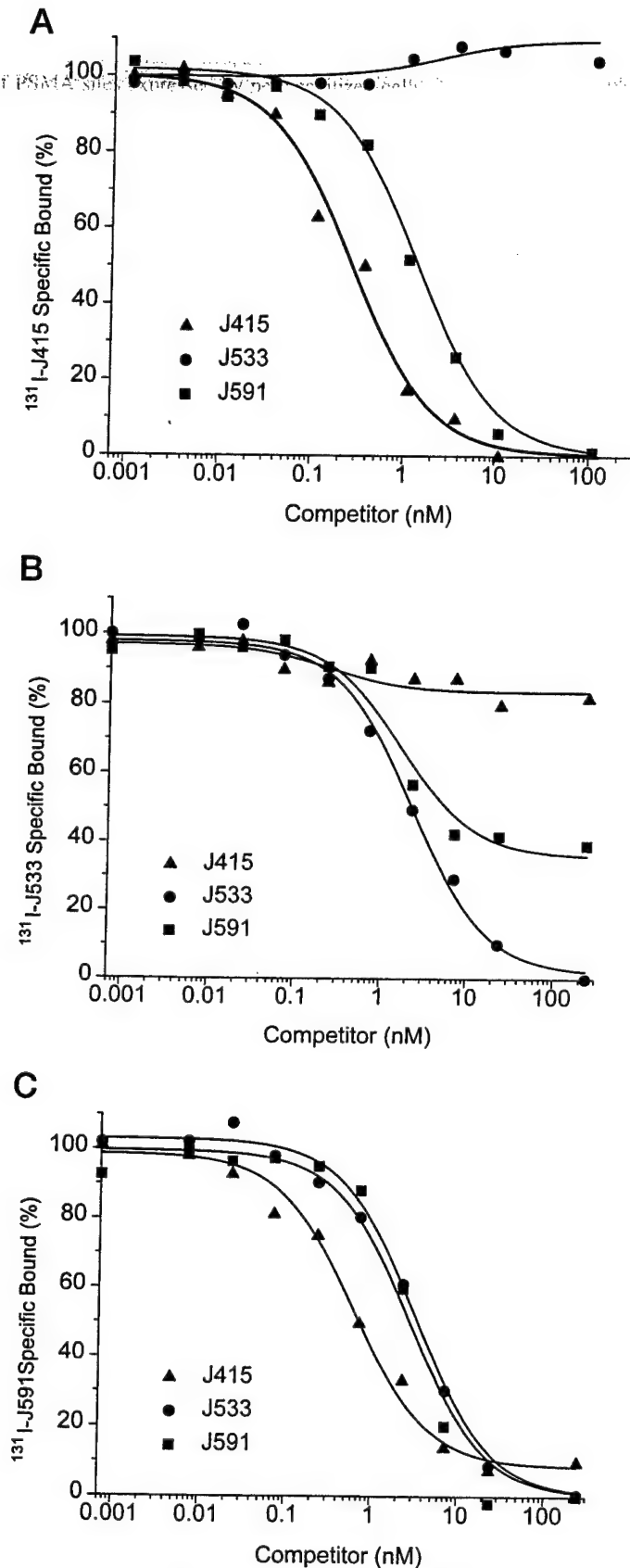


Fig. 4. Displacement binding of ^{131}I -labeled J415 (A), ^{131}I -labeled J533 (B), and ^{131}I -labeled J591 (C) to LNCaP cell membranes. The radioiodinated mAbs are incubated a fixed amount of LNCaP cell membranes in the presence of increasing concentrations of either J415 (▲), J533 (●), or J591 (■) at 37°C for 45 min. The membranes are then isolated by filtration through a glass fiber filter and then counted in a gamma counter. The amount of specific iodinated mAb bound is then plotted as a function of the increasing concentrations of the competing antibodies.

affinity (K_d s 1.76 ± 0.69 and 1.83 ± 1.21 nM), whereas J533 had a lower affinity (K_d 18 ± 5 nM). In parallel studies, all four mAbs bound to a similar number of PSMA sites expressed by permeabilized cells (1,000,000–1,300,000 sites/cell). In parallel studies performed with viable LNCaP cells, J415, J533, and J591 bound to a similar number of PSMA sites (*i.e.*, 600,000–800,000 sites/cell). In contrast, 7E11 specifically bound to only 10–15% of the PSMA sites expressed by apparently intact LNCaP Cells (K_d , 6.69 nM); but when the cells were deliberately ruptured (Fig. 6), 7E11 bound to a similar number of antigen sites as the other three mAbs. In parallel studies, using ^{131}I -labeled J591, permeabilized cells expressed about twice the amount of PSMA as intact LNCaP cells, suggesting that not all available PSMA is simultaneously expressed on the cell surface.

Internalization and Cellular Processing of J415 and J591. Both ^{131}I -labeled J415 and J591 demonstrated a poor cellular retention of

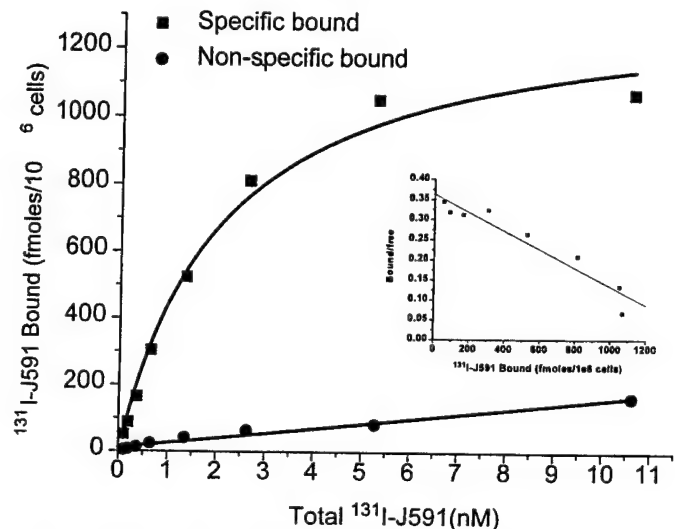


Fig. 5. Saturation binding of ^{131}I -labeled J591 to LNCaP cells. Increasing concentrations of ^{131}I -labeled J591 were incubated with intact LNCaP cells on ice for 60 min. Nonspecific binding (●) was determined in the presence of 100 nM unlabeled J591. Bound activity was isolated by centrifuging the cells and washing them twice with ice-cold buffer. Inset, Scatchard plot of the same data.

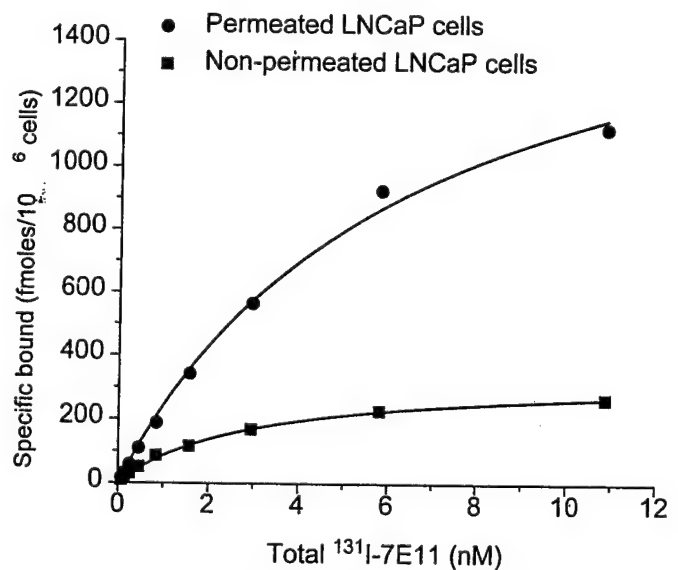


Fig. 6. Saturation binding of ^{131}I -labeled 7E11 to intact and ruptured LNCaP cells. Increasing concentrations of ^{131}I -labeled 7E11 were incubated with either intact (●) or permeabilized (■) LNCaP cells on ice for 60 min. Nonspecific binding was determined in the presence of 100 nM unlabeled 7E11. Bound activity was isolated by centrifuging the cells and washing them twice with ice-cold buffer.

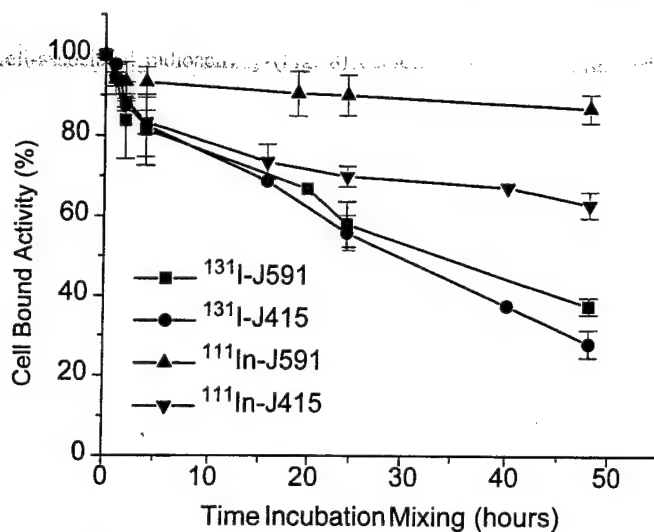


Fig. 7. LNCaP cell retention of radioiodinated J415 and J591. Petri dishes (8 cm²) with confluent LNCaP cells were incubated with 37 KBq of either ¹³¹I-labeled J591 (■), ¹³¹I-labeled J415 (●), ¹¹¹In-labeled J591 (▲), or ¹¹¹In-labeled J415 (▼). After 1 h at 37°C, the medium was removed, and the cells were washed once with fresh medium. One ml of fresh medium was added, and the cells were incubated for up to 2 days at 37°C. At various time points, the location and form of the radioactivity was determined. Bars, SD.

radioactivity (Fig. 7). For both mAbs, a biexponential curve fit of the data showed that ~10% of the radioactivity was released from the cells with an apparent half-life of 1 h, and the remaining 90% was released into the medium with apparent half-lives of 31 and 38 h for J415 and J591, respectively. In parallel studies, ¹³¹I-labeled J415 consistently showed a faster release of radioactivity than ¹³¹I-labeled J591. Little or no activity (<1%) was associated with the Triton X-100 (or NaOH) insoluble cell pellet. Analysis of the Triton X-100 soluble fractions indicated that there were no appreciable amounts of free ¹³¹I present (<1%). HPLC and TLC analysis of the culture medium showed that a large iodinated species, which corresponded to the same size as the intact mAbs, was being released from the cells, but this never amounted to >10% of the total activity, and after 4–6 h, no further release of this radioactive species was observed. The predominant metabolite of the iodinated mAbs found in the cell medium had the same HPLC and TLC elution profile as free ¹³¹I⁻. Several studies compared ¹³¹I-labeled J591 and DOTA-J591, and no significant differences in the retention of ¹³¹I by the cells were noted between the two forms of the same mAb. In all of the studies performed, no increase in cell death was noted as compared with the control groups that received no radiolabeled antibodies.

In comparison to the iodinated mAbs, the ¹¹¹In-labeled DOTA-J415 and DOTA-J591 demonstrated a high cellular retention of radioactivity (Fig. 7). For ¹¹¹In-DOTA-J415, a biexponential curve fit of the data showed that ~20% of the radioactivity was released from the cells with an apparent half-life of 2 h, and the remaining 80% was released into the medium with an apparent half-life of 160 h. For ¹¹¹In-DOTA-J591, the cellular release of ¹¹¹In species was much slower, and a biexponential curve fit of the data showed that about 5–10% of the radioactivity was released from the cells with an apparent half-life of 1 h, and the remaining 90–95% was being released into the medium with an apparent half-life of 520 h. Little or no activity (<1%) was associated with the Triton X-100 (or NaOH) insoluble cell pellet. HPLC and TLC analysis of the cell medium showed that a large ¹¹¹In species, which corresponded to the same size at the intact mAbs, was being released from the cells, but this never amounted to >10% of the total activity, and after 4–6 h, no further release of this radioactive species was observed. For both J415 and

J591, two main ¹¹¹In-labeled metabolites were observed in the medium. Analysis of the cell-associated radioactivity (Fig. 8) demonstrated the rapid formation of two groups of metabolites (based on molecular size). One group of metabolites achieved a maximum concentration after 1–2 h, after which it began to steadily decline. The second group of metabolites, however, demonstrated an ever-increasing intracellular concentration. This second metabolite did not behave the same as ¹¹¹In³⁺ (HPLC or TLC), but rather it had a similar molecular weight as an ¹¹¹In-DOTA or an ¹¹¹In-DOTA-amino acid fragment. The first metabolite had a molecular weight between that of the intact mAb and the second metabolite (*M_r* ~10,000–30,000).

The uptake rates of ¹¹¹In-labeled J415, J591, and 7E11 by LNCaP cells showed a similar initial uptake rate for J415 and J591, which was 10–20 times faster than that of 7E11 (Fig. 9). However, by 4 h after the addition of the radioactivity, the cells treated with ¹¹¹In-DOTA-J591 have incorporated and retained more activity than those treated with ¹¹¹In-DOTA-J415.

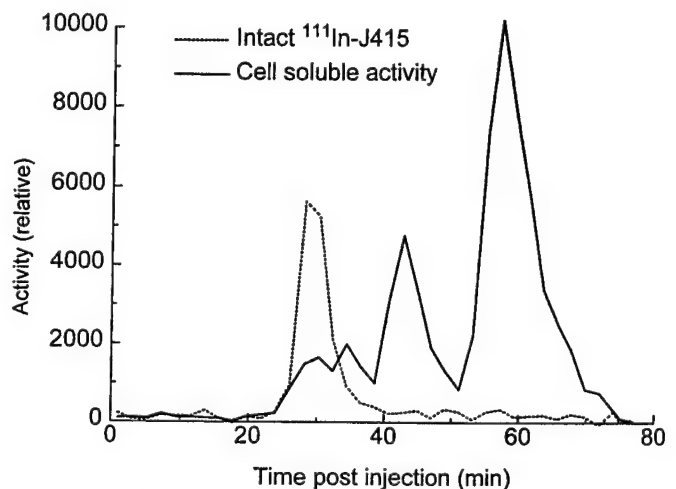


Fig. 8. HPLC chromatograms of ¹¹¹In-DOTA-J415 and radioactivity recovered from LNCaP cells at 48 h after incubation. The intact ¹¹¹In-DOTA-J415 elutes at 28 min after injection, and two main metabolites elute at 43 and 57 min after injection, respectively.

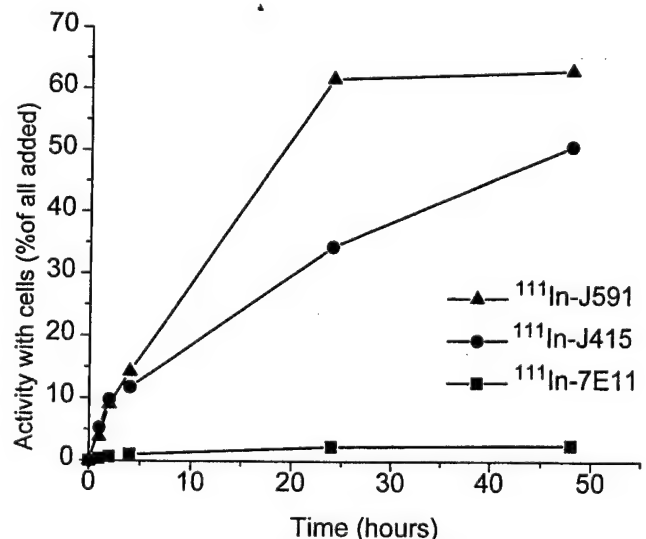


Fig. 9. Incorporation of ¹¹¹In-labeled cells by LNCaP cells. Petri dishes (8 cm²) with confluent LNCaP cells were incubated with 37 KBq of either the ¹¹¹In-DOTA-J415 (●), ¹¹¹In-DOTA-J591 (▲), or ¹¹¹In-DTPA-7E11 (■). The samples were incubated at 37°C, and at various time points the location and the amount of cell-associated radioactivity were determined.

Table 1. Inhibition constants for unlabeled J415, J533, J591, and 7E11 for the binding of ^{131}I -labeled J415, ^{131}I -labeled J533, and ^{131}I -labeled J591 binding to PSMA expressed by LNCaP cell membranes

Iodinated mAb	IC ₅₀ s for displacing mAb (nM)			
	J415	J533	J591	7E11
^{131}I -labeled J415	1.5 ± 0.9	ND ^a	6.6 ± 4.5	ND
^{131}I -labeled J533	ND	2.3 ± 1.5	1.7 ± 1.3	ND
^{131}I -labeled J591	1.3 ± 0.9	7.7 ± 5.5	3.1 ± 1.5	ND

^a ND, not displaced.

DISCUSSION

To understand the characteristics of and to select the best imaging/therapeutic agent, we studied binding characteristics and retention rates of the variously labeled mAbs using the LNCaP cell line, which expresses PSMA (Table 1).

The initial labeling of the three mAbs with ^{131}I , up to a specific activity of 350 MBq/mg, resulted in little or no apparent loss of immunoreactivity. Similarly, the conjugation of up to an average of five DOTA chelates per mAb enabled specific activities of up to 280 MBq ^{111}In /mg DOTA-J591 with no apparent loss of immunoreactivity. Site-specific modification of the antibody is sometimes required when this type of random DOTA coupling results in loss of immunoreactivity attributable to the presence of a lysine residue in the antigen binding domain. High specific activities are often required for accurate mAb characterization and particularly when large amounts of the radiotherapeutic agent are administered to a patient. J415 and J591 could be modified with sufficient DOTA to produce high specific activity ^{111}In - and ^{90}Y -labeled mAbs for both *in vitro* binding studies and eventual *in vivo* studies.

Early approaches to labeling mAbs with radiometals used DTPA, which in its dicyclic anhydride form could be conveniently coupled to mAbs (22). Unfortunately, this simple coupling chemistry produced a more labile chelate than bifunctional forms of the same unconjugated DTPA chelator (23). Macrocyclic chelators have shown even higher kinetic stability (24), but they are even more time consuming to chemically synthesize (25). DOTA can be coupled directly to mAbs using simple chemistry and commercially available materials (20).

The reaction kinetics for ^{111}In and DOTA are longer than for DTPA, but an incubation period of 15 min can give high labeling yields. The DOTA chelator was immensely superior to DTPA in its ability to tightly chelate ^{111}In in the presence of an excess of competing ligand. This is in agreement with other studies (20, 26) and underlies the importance of using stable chelates with mAbs that can stay in circulation for prolonged periods of time in the presence of competing ligands (e.g., transferrin). The higher stability of the ^{111}In -labeled DOTA complex relative to the ^{111}In -labeled DTPA complex also applies for the ^{90}Y complex (20, 26) and is an important prerequisite for radiolabeled mAbs used for either diagnosis or therapy because optimal tumor:nontumor ratios are often achieved after 2–4 days. Because ^{111}In -DOTA-J591 is stable to DTPA competition, it enables nonspecifically bound ^{111}In to be removed by challenging with DTPA and a simple column separation to yield a highly pure radiopharmaceutical.

High binding affinity between the mAb and the target antigen is another prerequisite to *in vivo* targeting of tumor antigens. The binding studies with the iodinated mAbs showed that two of three of these mAbs against PSMA_{ext} (i.e., J415 and J591) and 7E11 have similar nanomolar binding affinities. The use of intact and ruptured cells showed clearly that ^{131}I -labeled 7E11 binds to the intracellular domain of PSMA, consistent with other reports (13, 17). There was some binding of 7E11 to "intact" LNCaP cells, but that could be explained by the presence of a small population of cells ruptured during the

trypsinization of the cells from the cell culture flasks and subsequent handling during resuspension. Because J591 recognized and specifically bound to twice the number of PSMA sites in permeabilized cells as opposed to intact cells, this suggests that only 50% of all cellular PSMA is exposed extracellularly. Also, 10–15% binding of 7E11 could be explained by the presence of a population of 5–7% of permeabilized cell in the "intact" cell preparation. This was confirmed in other studies that examined the binding and cellular uptake of the mAbs with plated LNCaP cells and showed a 7E11 uptake of 3–4% that of J415 and J591. This quantitative difference seen might explain why one group claims that the 7E11 binds to apparently intact LNCaP cells (27), whereas other groups report no such binding (13, 17, 18). Additionally, the Barren study (27) used cells that were scrapped from the monolayer, a procedure known to create significant cell rupture.

Effective systemic targeting of tumors has been achieved with both iodinated and metalchelated antibodies. A significant factor in selecting between the two approaches is in whether the antibody is internalized. In the case of an internalizing antibody, directly iodinated antibodies are metabolized within the cell, and the main metabolites of iodide and iodotyrosine are then freely released from the cell. Conversely, the metal chelate-labeled antibody is metabolized to leave a chelate-amino acid fragment that is typically not released from the cell and is trapped for further degradation. This effect can produce vastly different residence times for two differently labeled forms of the same antibody (28). In the LNCaP cell model, PSMA is known to be an internalizing surface bound glycoprotein (18). These studies clearly support the notion that metallo-labeled mAbs are superior for cells expressing internalizable PSMA and that metabolites from the DOTA-mAb conjugate are not appreciably released from LNCaP cells.

In conclusion, both J415 and J591 have similar nanomolar affinities to PSMA as 7E11. Similarly, these two mAbs are far more readily bound and were internalized by live LNCaP cells than 7E11. The ^{111}In -labeled DOTA conjugates are able to associate more radioactivity with LNCaP cells than the comparable iodinated forms. The ^{111}In -labeled DOTA conjugates are also more stable to loss of ^{111}In than DTPA-7E11. These findings make DOTA-J415 and DOTA-J591 attractive candidates for further evaluation as either diagnostic or radiotherapeutic agents in patients with various cancers that express PSMA.

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APPENDIX- B

Figure 1: Tumor/Blood and Tumor/Muscle Ratios of ^{131}I and ^{111}In -Labeled
Mabs (J591, J415, 7E-11) in Nude Mice with LNCaP tumors

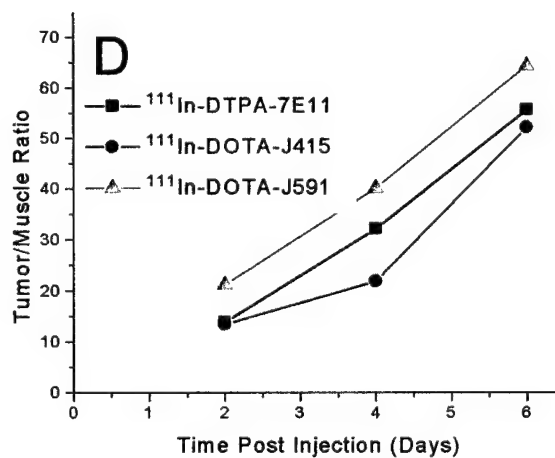
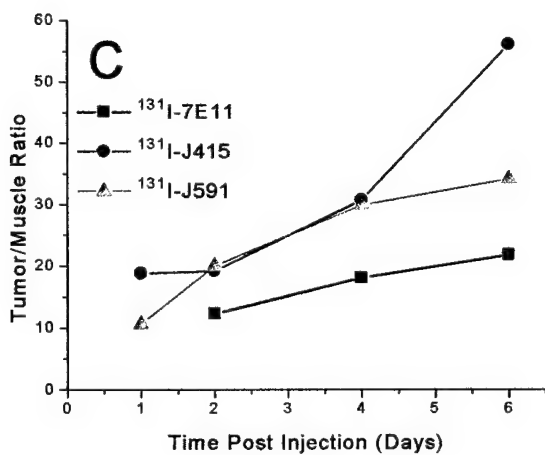
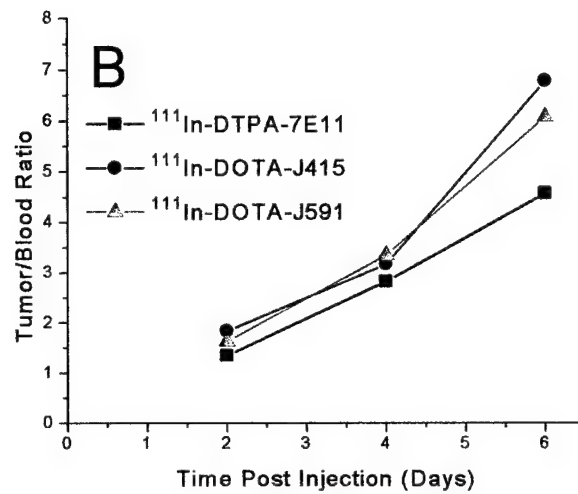
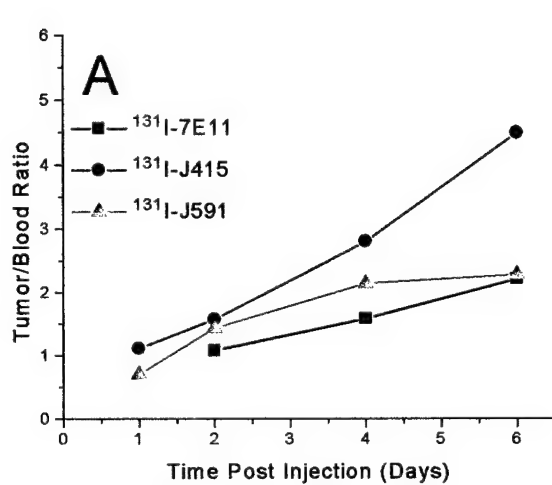


Figure 2: Gamma Camera Images of a Nude Mouse with LNCaP tumor.
Images were obtained with a pin-hole collimator on days 1,2,3,4, and
6 post injection of ^{111}In -DOTA-J591.

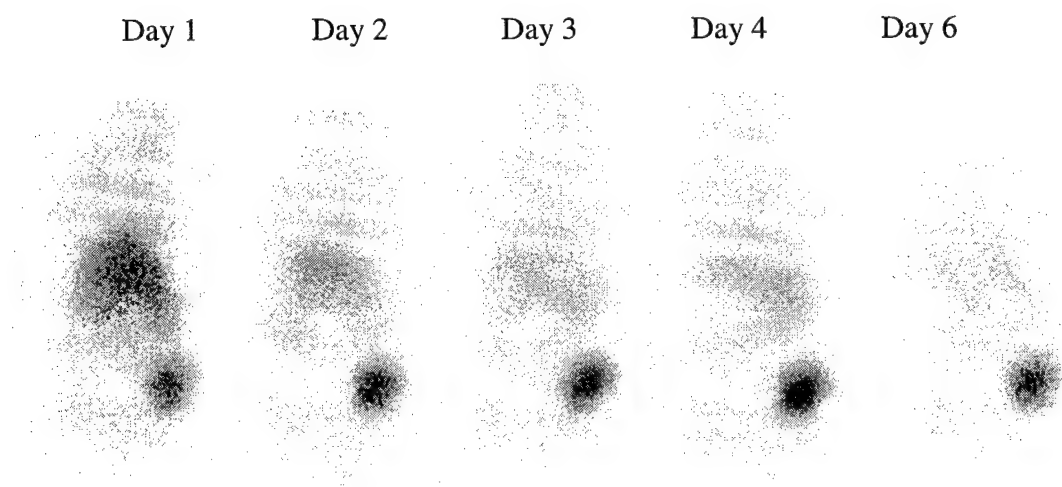


Figure 3A: Blood Clearance of Radiolabeled J591 MAb.

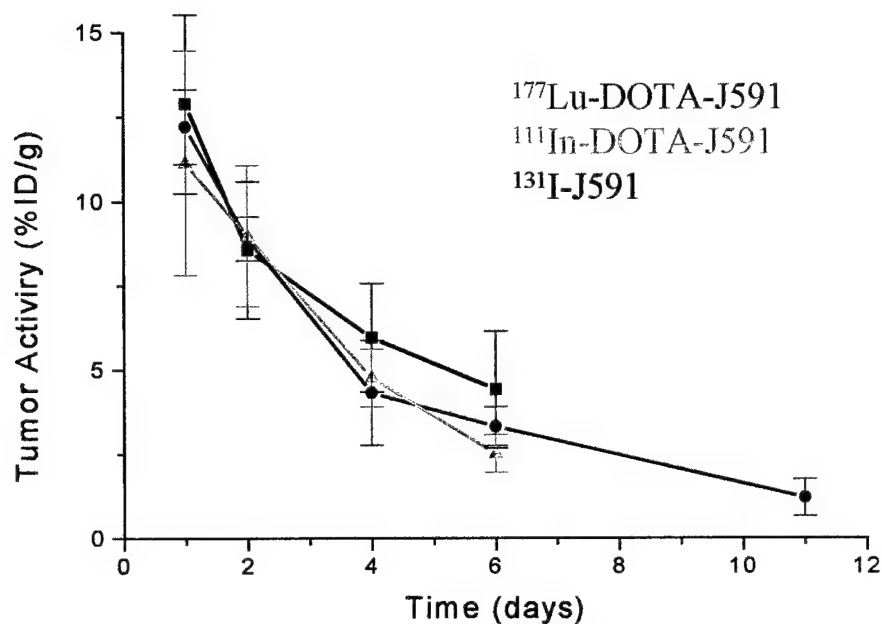


Figure 3B: Tumor Uptake of Radiolabeled J591 MAb.

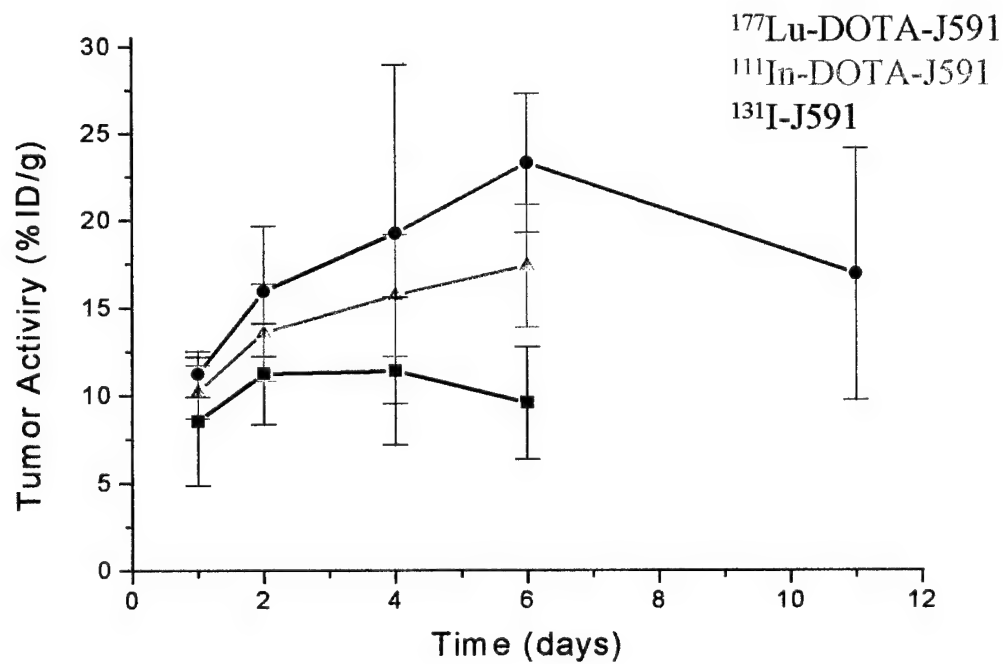


Figure 4: Radioimmunotherapy of nude mice bearing LNCaP tumors using ^{131}I -J591 MAb.

Compared to untreated controls, anti-tumor effect of ^{131}I -J591 was dependent on the dose. At $100\mu\text{Ci}$ dose, there was some delay or reduction in tumor size. But at $300\mu\text{Ci}$ dose, all the mice had a significant reduction in tumor size. 2/5 mice in control group died in 4 weeks and the remaining 3 survived more than 6 weeks. With I-131, while there was reduction in tumor size, the % survival also decreased compared to controls. Interestingly, mice that received $100\mu\text{Ci}$ intraperitoneally survived longer than 6 weeks.

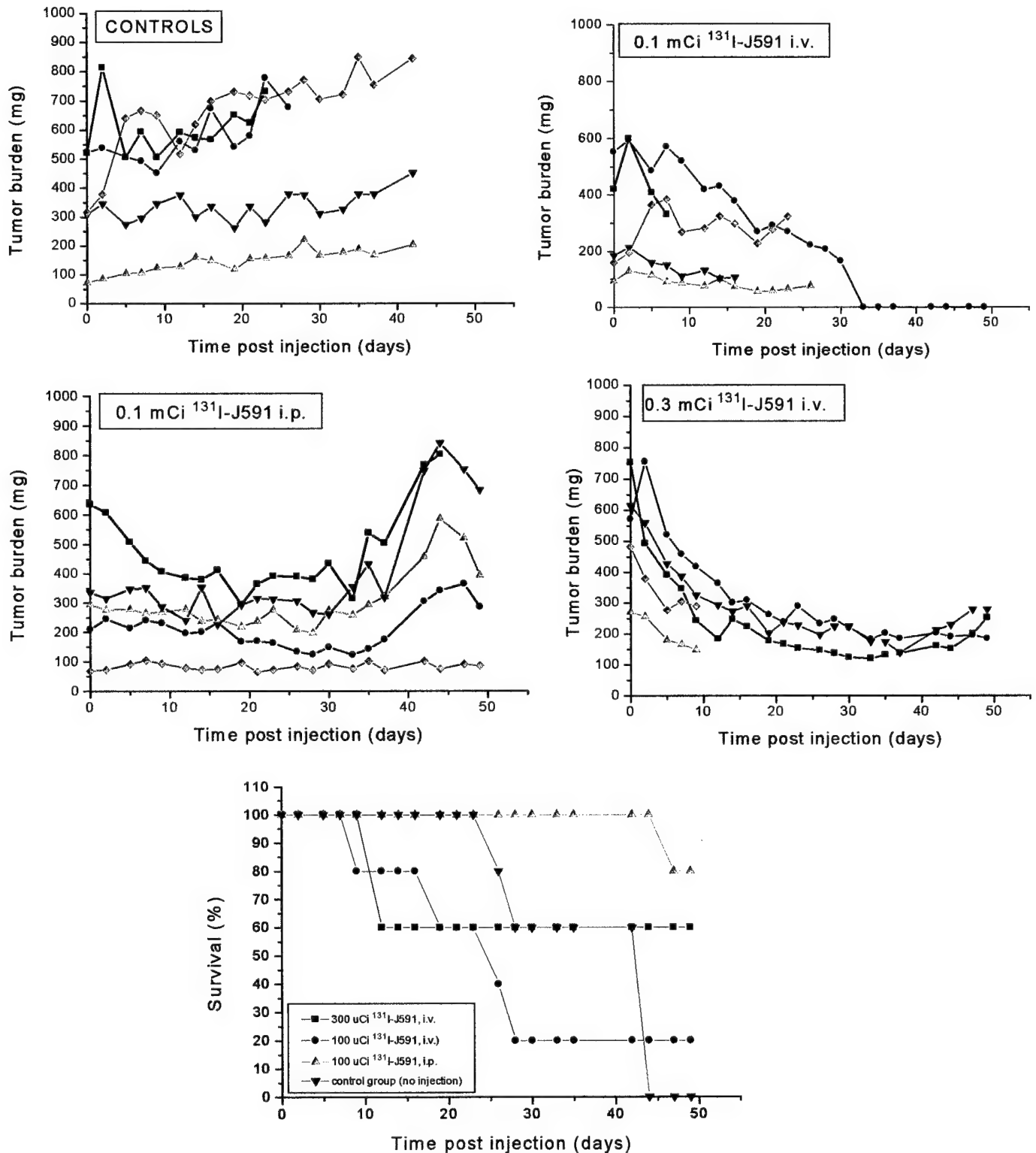


Figure 5A: Radioimmunotherapy of nude mice bearing LNCaP tumors: ^{131}I -J591 Vs. ^{90}Y -J591 MAb.

(5-7 mice/ group; 4 groups total)

Compared to untreated controls, the anti-tumor effect of ^{90}Y -J591 (35 μCi) was similar to that of ^{131}I -J591 (300 μCi). Mice that received a second injection of ^{90}Y -J591 (35 μCi) showed further reduction in tumor size.

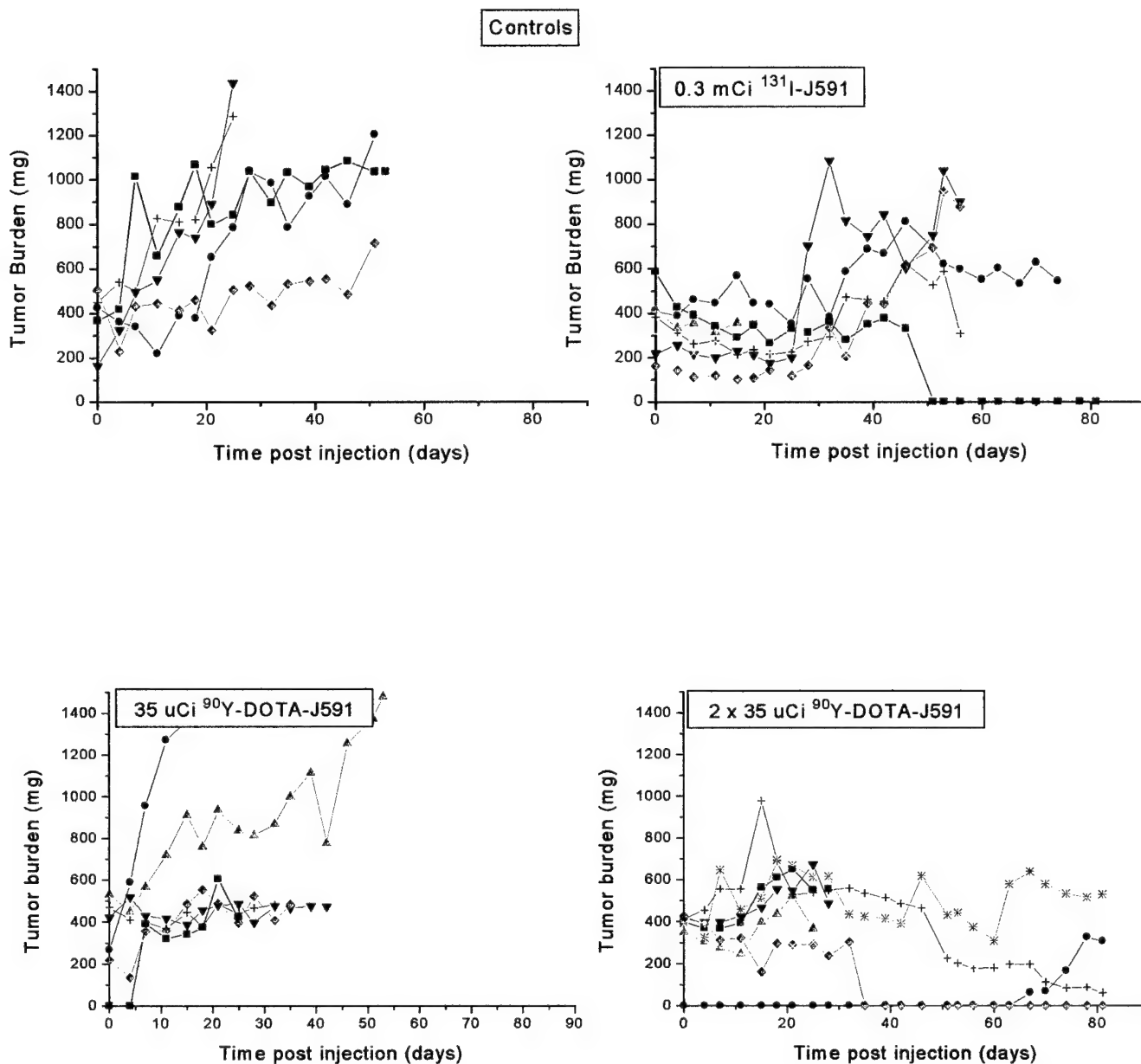


Figure 5B: Treatment of nude mice with LNCaP tumors using ^{90}Y -DOTA-J591:**Effect on total body mass (weight of the animal)**

There was a gradual reduction (20% within 6-8 weeks) in the total body mass of mice both in control group and mice that received only 30 μCi of ^{90}Y -DOTA-J591. However, following second injection of the treatment (at the same dose level), there was a gradual recovery of total body mass. With ^{131}I -J591 (at 300 μCi), there was some recovery of body mass, but most of the animals died within 8-10 weeks.

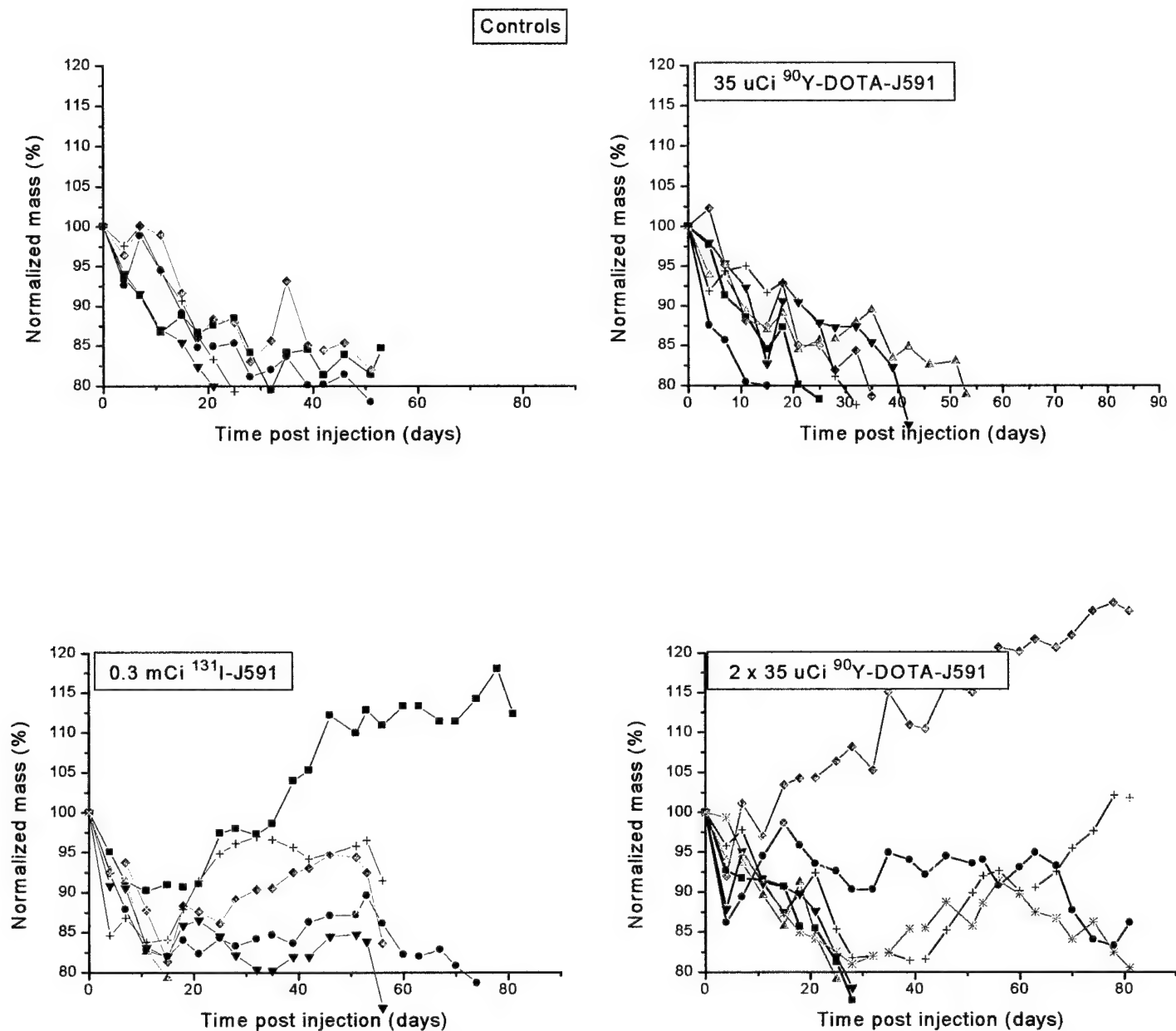


Figure 6 A: Radioimmunotherapy of nude mice bearing LNCaP tumors using ^{90}Y -DOTA-J591 MAb.
(Comparison with untreated controls and mice treated with non-specific antibody (F23))

Mice were treated with 3 different doses (30, 60 and 90 μCi) of ^{90}Y -DOTA-J591 MAb. The mice in each group received repeat injections at the same dose level 3 times (days 0, 28 and 56). Mice in control group also received non-specific antibody 3 times. Total 5 groups; 6-7 mice/group

The antitumor effect of ^{90}Y -DOTA-J591 MAb was dose dependent (greatest response at 90 μCi (Figure 6B)) and very specific since control mice (that received non-specific antibody) had an uncontrolled tumor growth.

Compared to control groups, mice that received 30 and 60 μCi of ^{90}Y -DOTA-J591 MAb had a significantly longer survival rate (Figure 6B)

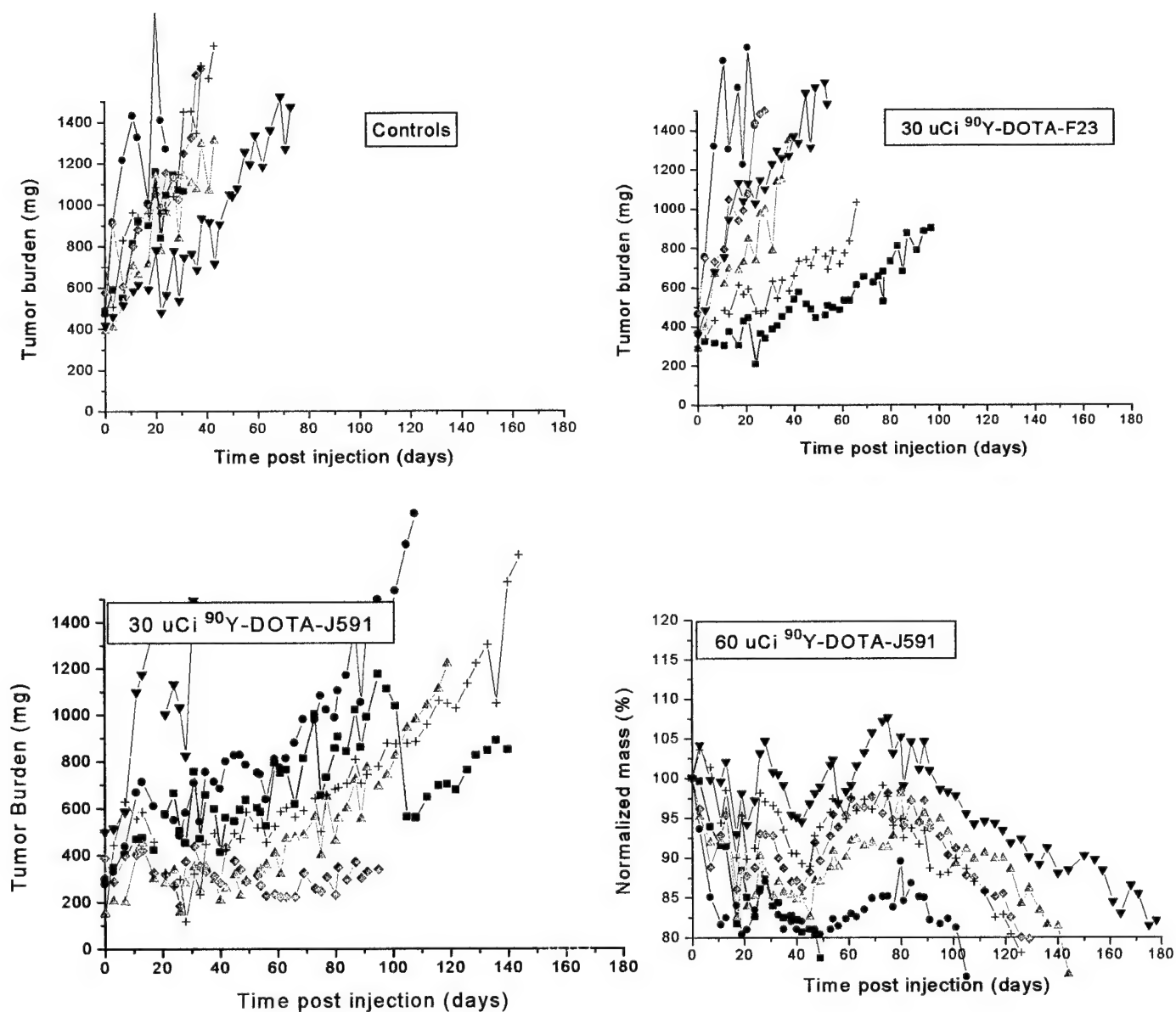


Figure 6 B: Radioimmunotherapy of nude mice bearing LNCaP tumors using ^{90}Y -DOTA-J591 MAb.
(Comparison with untreated controls and mice treated with non-specific antibody (F23))

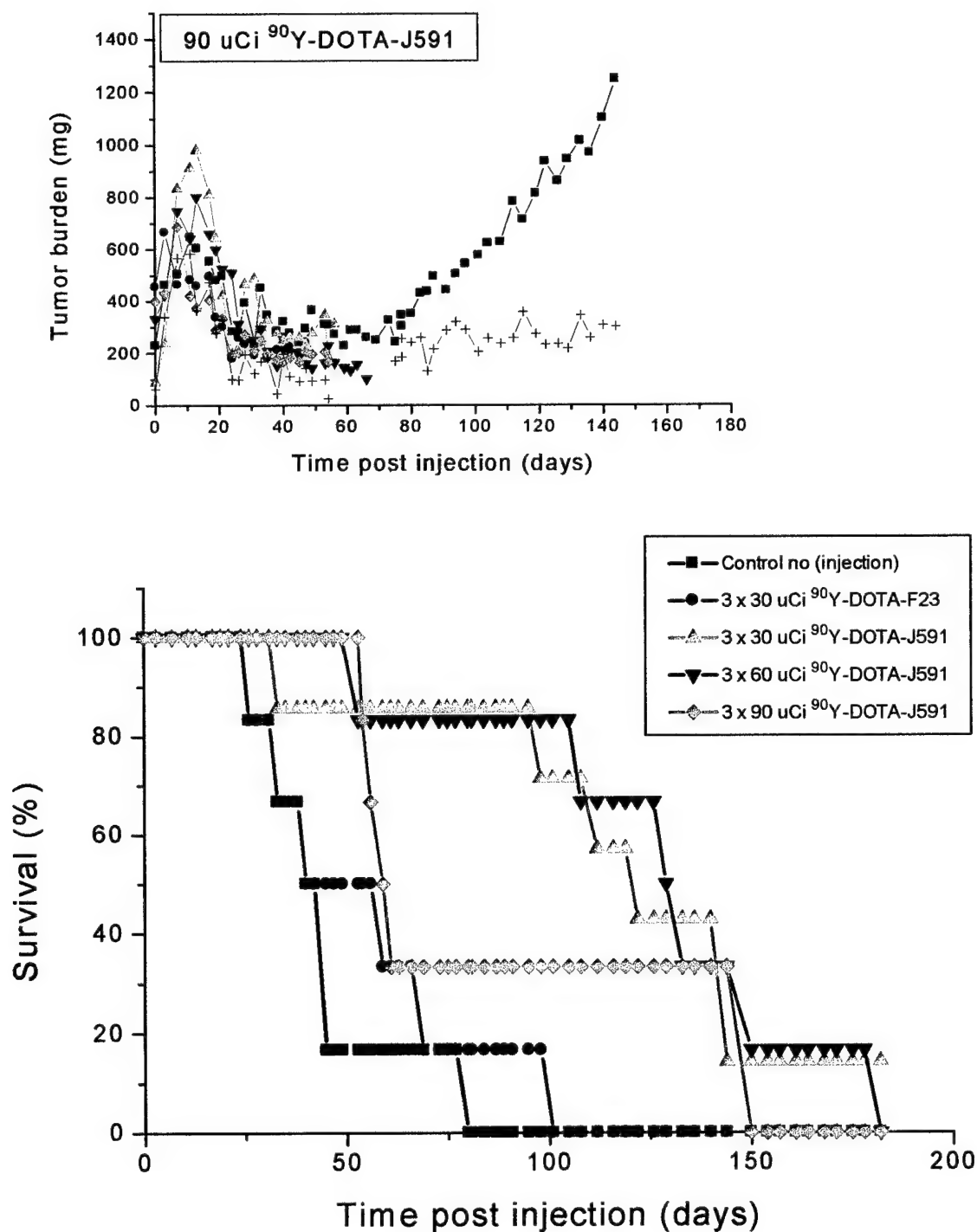


Figure 6C: Treatment of nude mice with LNCaP tumors using ^{90}Y -DOTA-J591:**Effect on total body mass (weight of the animal)**

There was a gradual reduction (20% within 6-8 weeks) in the total body mass of mice both in control group. Mice that received repeated (days 0, 28, and 56) treatment doses of 30 and 60 μCi of ^{90}Y -DOTA-J591 showed a gradual increase in total body mass compared to control mice.

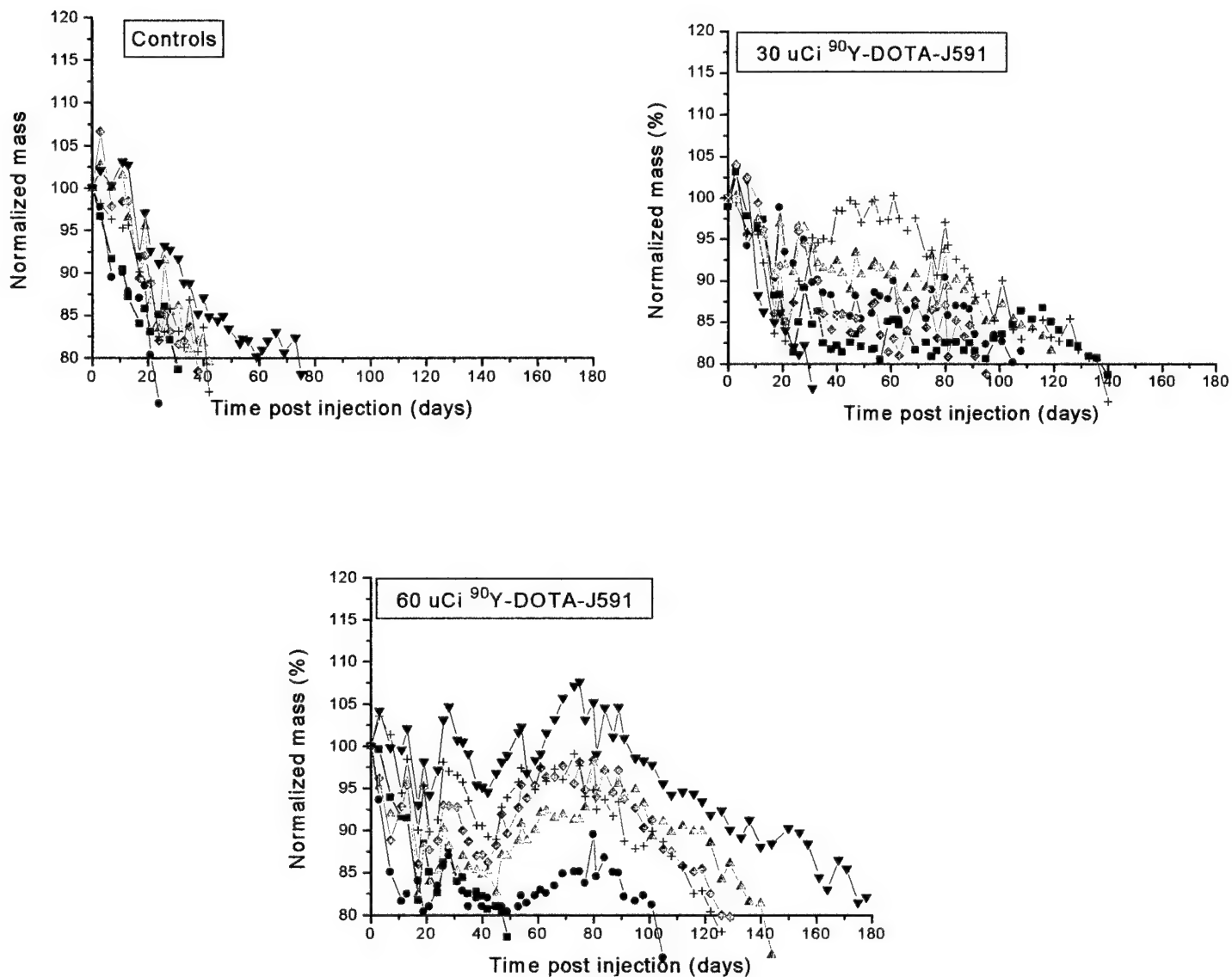


Figure 7: Radioimmunotherapy of nude mice bearing LNCaP tumors using ^{90}Y -DOTA-J591

Dose-response (anti-tumor effect) relationship and relative toxicity.

3 groups ($n=4-5$) of mice were injected with different doses (150, 200, and 300 μCi) of ^{90}Y -DOTA-J591. There was a significant reduction in tumor volume with all 3 dose levels. However, at 150 μCi dose level, 5 weeks after the treatment dose, tumors started to grow again (tumor volume increased 2-times the baseline value). In contrast, most of the mice at 200-300 μCi dose level died within 3-4 weeks following the treatment dose suggesting that these dose levels are associated with increased toxicity.

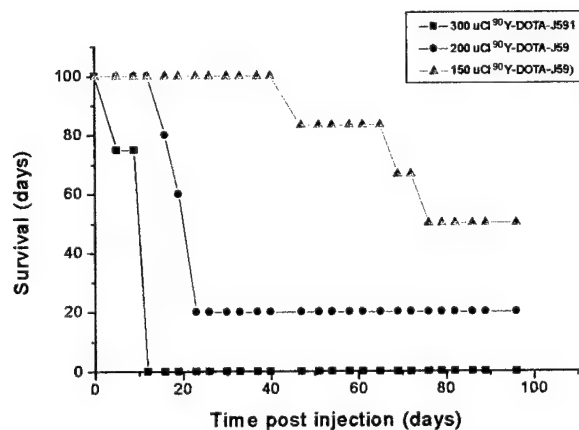
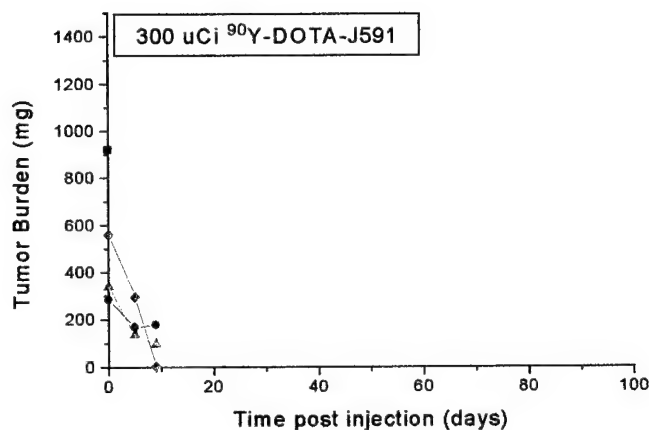
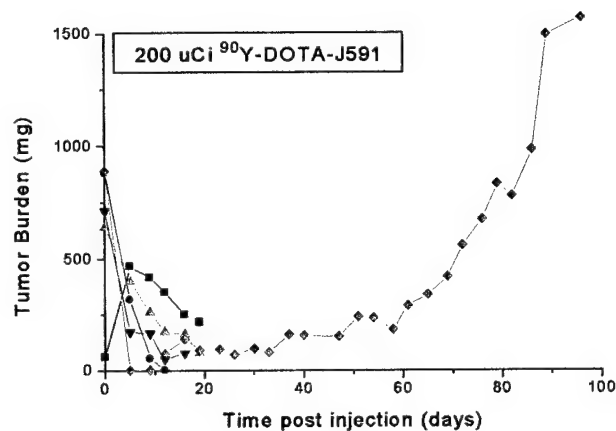
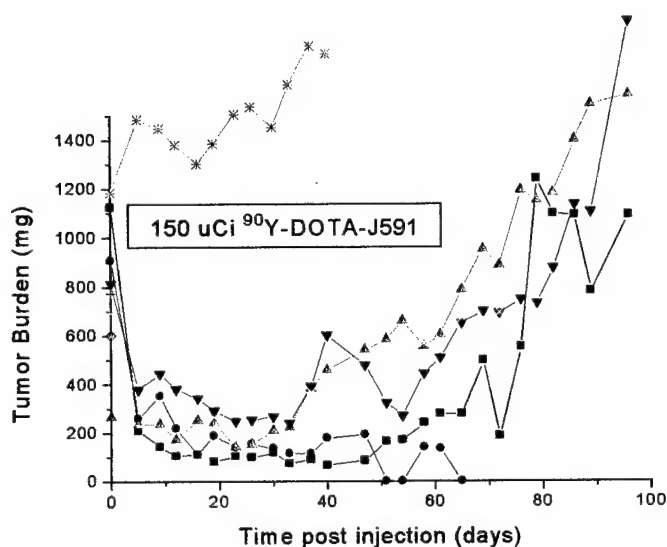


Figure 8: Radioimmunotherapy of nude mice bearing LNCaP tumors: **^{90}Y -DOTA-J591 Vs. ^{90}Y -DOTA-J415**

The anti-tumor effect of ^{90}Y -DOTA-J591 was compared to that of ^{90}Y -DOTA-J415. Two groups (n=7/group) of mice received 60 μCi of labeled MAb. Mice in each group received a total of 3 doses (at the same dose level) over a period of 2 months (Days 0, 35 and 63). For comparison, a control group received no treatment.

Compared to controls, both antibody (J591 and 415) preparations had a significant anti-tumor response. However, ^{90}Y -DOTA-J591 response appeared to be greater and more consistent. In addition, the % survival with Y-90 labeled J591 and J415 was significantly greater compared to that of controls.

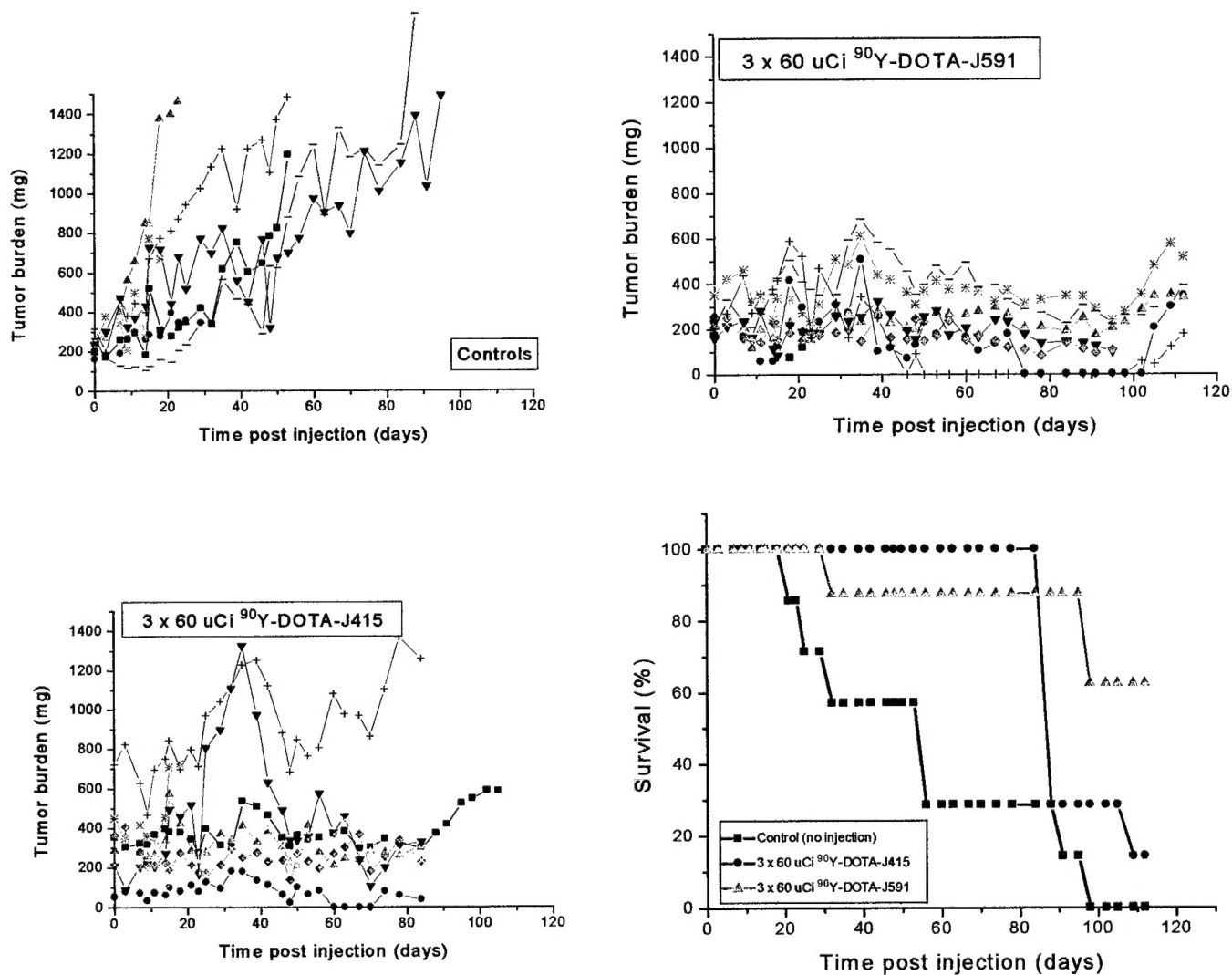


Figure 9A: Radioimmunotherapy of nude mice with LNCaP tumors using ^{177}Lu -DOTA-J591.

3 groups (n=6-7/group) of mice were injected with a single dose of 100, 200, and 400 μCi of ^{177}Lu -DOTA-J591. A control group (n=7) received no treatment. With 100 μCi , there was a delay in tumor growth. But at 200-400 μCi dose levels, the anti-tumor effect was significant. 6-8 weeks following treatment, tumors started growing slowly. There was greater survival rate at 200 μCi and is less toxic.

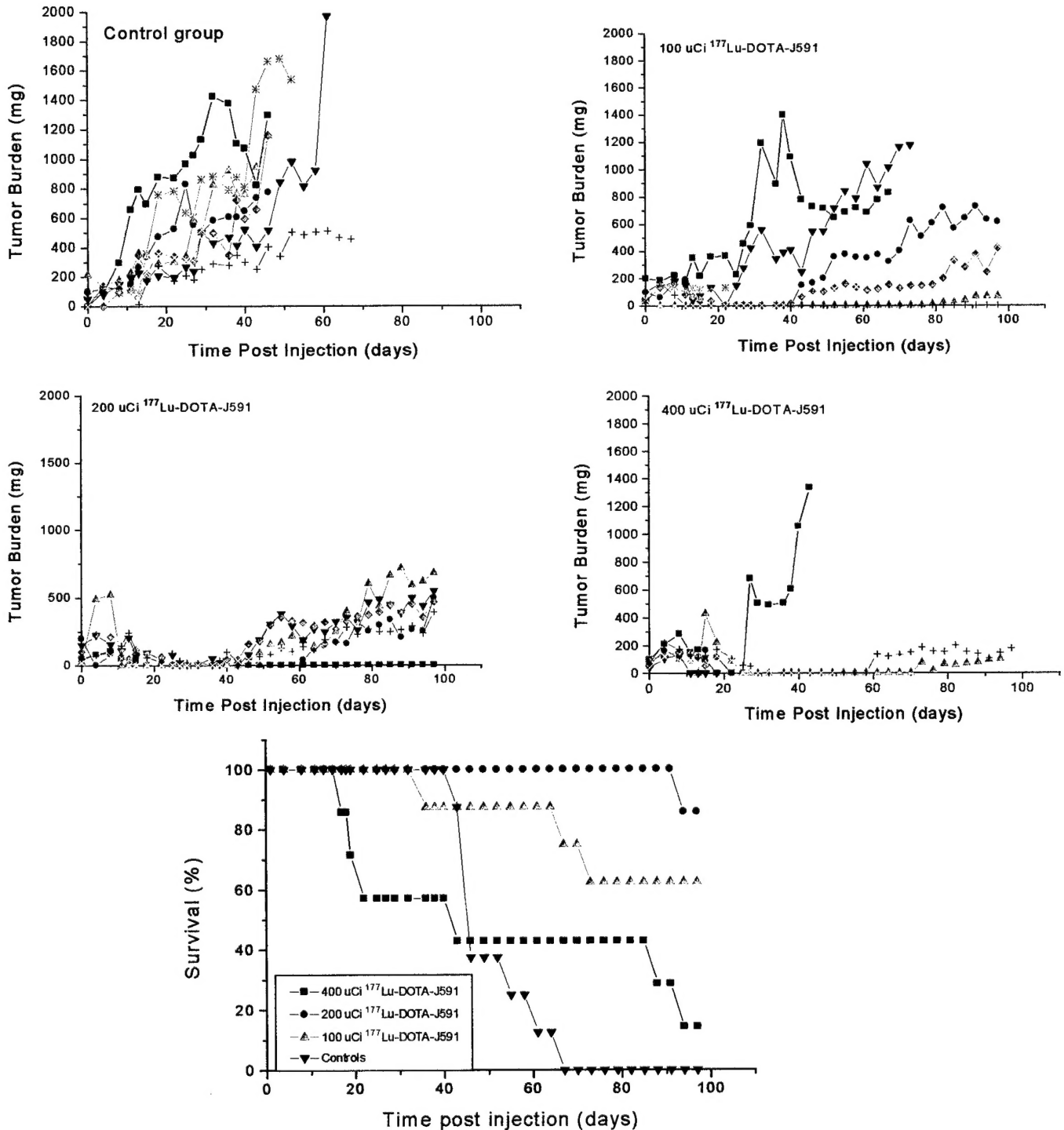
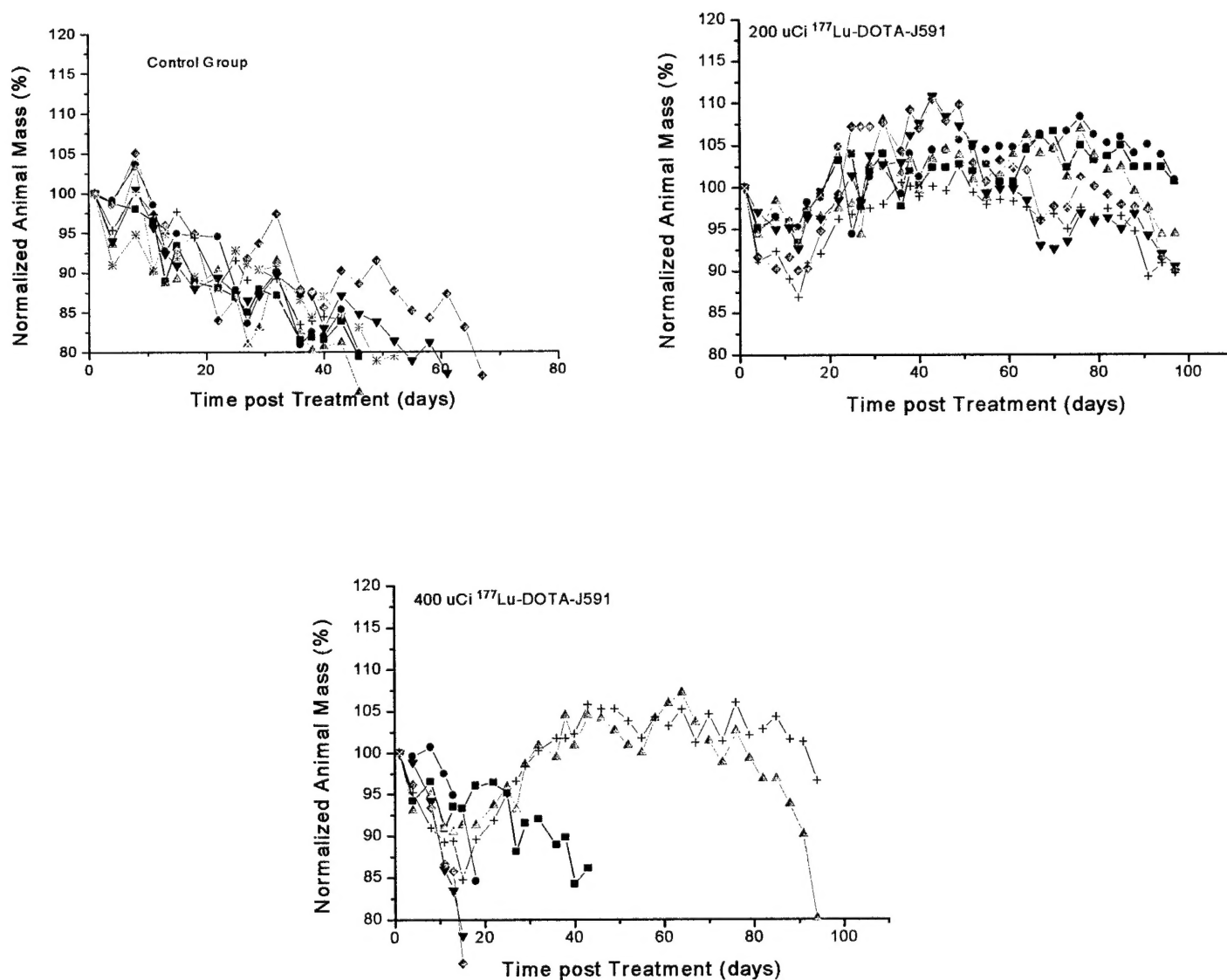


Figure 9B: Treatment of nude mice with LNCaP tumors using ^{177}Lu -DOTA-J591:**Effect on total body mass (weight of the animal)**

There was a gradual reduction (20% within 6-8 weeks) in the total body mass of mice in the control group. In contrast, mice treated with 200 μCi of ^{177}Lu -DOTA-J591 there was no significant reduction in the body mass over a period of 8-10 weeks. However, at 400 μCi dose level 50% of the mice had a gradual decline in the body mass and died within 3-6 weeks.



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